THE QUALITY OF THE DIET AND THE CONSUMPTION OF SUCROSE SOLUTIONS[1]

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(Received for publication June 16, 1947)

In 1939 the national expenditure for soft drinks was about $400,000,000 (Reid, '43). The following studies of the past 4 years have attempted to use the white rat to determine some of the factors that influence the consumption of these beverages which consist chiefly of a 10% solution of sugar with flavors, coloring matter and acids such as phosphoric and citric. Previous studies have already been published in regard to the etching of the enamel of the teeth by these beverages (Bieri, McCay, Restarski and Gortner, '46).

EXPERIMENTAL

The water consumption of the white rat

Eleven rats were used to measure the water consumption when fed our stock diet.[2] Calibrated glass tubes were used for these measurements. Rats were about half grown at the start of the study. Six studies were made covering the months of August, September and October. The results are summarized in table 1.

[1] These experiments were started at the Naval Medical Research Institute, Bethesda, Maryland, and concluded in the Animal Nutrition Laboratory, Cornell University, Ithaca, New York. Assistance in the earlier studies was given by June Sullivan and R. A. Gortner, Jr. This research was financed in part by a grant from the Office of Naval Research to determine factors responsible for the deterioration of gums, teeth and vestment tissues.

[2] G.L.F. Dog Meal produced in Canandaigua, New York.

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Attempts were made to correlate these data with humidity and temperature but the numbers were small and no correlation was found. Individual rats are quite constant in their daily water consumption but vary widely among themselves. The study indicated some sex difference but this was not confirmed in subsequent studies.

TABLE 1

Daily water consumption per 100 gm of live weight for 11 rats of each sex.

PERIOD 1943	FEMALE			MALE			MEAN DAILY TEMP.	
	Mean	Max.	Min.	Mean	Max.	Min.	Wet bulb	Dry bulb
	ml	*ml*	*ml*	*ml*	*ml*	*ml*	°*F.*	°*F.*
Aug. 5–17	14.7	21.6	10.9	12.9	19.7	9.7	73	84
Aug. 18–27	12.8	14.7	10.4	12.3	24.6	8.8	68	74
Aug. 28–Sept. 8	14.0	16.6	12.0	12.4	24.5	9.2	71	73
Sept. 9–30	14.5	17.8	10.2	12.2	22.0	9.2	62	71
Oct. 5–15	15.3	22.2	9.4	11.4	16.5	8.7	63	73
Oct. 16–27	16.6	22.8	10.6	11.9	17.1	8.2	63	73
Mean and P.E.M.[1]	14.65 ± 0.27			12.2 ± 0.34				
Difference between means	2.45 ± 0.43							
Mean increase of body wt. (gm): Female, 143–186; Male, 151–242								

[1] Probable error of the mean.

The consumption of 10% sugar solutions

In the course of studying the etching effects of acid beverages upon the teeth of different species, it was observed that mice, rats, hamsters, monkeys and guinea pigs are fond of sugar solutions but many dogs drink them with great reluctance. Hausmann ('33) found the rat to be fond of a solution sweetened with saccharine. Richter and Campbell ('40) discovered that the rat preferred 10% sugar solutions. All of our studies have used this concentration.

In order to compare the relative appeal of 10% sucrose solutions and distilled water sweetened with saccharine and dulcin, 2 groups of rats containing 7 each were placed in large

stock cages. These rats had a mean weight of 225 gm and were 3 months old at the beginning of the study. The solution of saccharine contained 7 mg per 100 ml and the dulcin 30 mg per 100 ml.

In the first trial the groups of rats were allowed to choose between 4 tubes containing water, 10% sucrose, saccharine and dulcin during a period of 20 days. The mean daily consumptions, in ml per rat, were water 3, sucrose 60, saccharine 9, and dulcin 2.

In a second study with the same animals for the same period, the sugar solution was omitted. The daily consumptions in this experiment were water 3, saccharine 30, and dulcin 8.

A third study compared water with dulcin. The mean daily consumptions were water 8 ml, and dulcin 20.

A fourth comparison involved water and saccharine and yielded the values of 9 and 26, respectively.

These data indicate that, when our stock diet was used, the rat's preference was in the following order: sucrose, saccharine, dulcin and water. These results might have been modified by varying the concentration of the synthetic sweetening agents but this was not done.

In exploratory studies for further work comparison was made of the excretion of urine by rats drinking water in contrast to sucrose solution. For a month comparison was made using 2 rats weighing 360 gm. During this period rat A consumed daily, 72 ml of sucrose solution and 10 gm of stock diet. The daily urine excretion was 43 ml. In comparison rat B, drinking water, ingested 40 ml of water, ate 17 gm of feed and excreted 15 ml of urine, daily.

Likewise an initial study was made to determine the relative amounts of sucrose solution and water that were drunk by female rats nursing large litters. In nursing a litter of 12, 1 female consumed daily 69 ml of sucrose and 17 ml of water while a second female nursing a litter of 10 drank daily, 71 ml of the sucrose solution and 24 ml of water.

The relative consumption of sucrose solution and water as a rat grows

A series of exploratory studies using 140 rats was made to determine if the growing rat would tire of the daily ingestion of sucrose solutions, or sucrose-phosphoric acid as found in cola beverages. Paired rats were used to compare the relative amounts of water and sucrose solution consumed at different periods from weaning until the rats were 100 days old. In part of the trials, the rats were not used until they weighed 100 gm.

Detailed data from these studies are not presented since the results were the same in each trial. A period of 5–10 days was required for most rats to develop their taste for sucrose solutions, so that daily performance was consistent. After this the amount drunk increased regularly until the experiment terminated after 70 days.

In one typical study 28 rats were divided into 2 groups. One group was given sucrose solution and the other group water. The animals were caged individually. During the first 28 days the mean water consumption per day was 22 ml. Per 100 gm of body weight per day it was 19 ml. During the next 42 days the comparable values were 21 ml per day and 12 per 100 gm of live weight.

During this same period those given sucrose consumed during the first period a volume each day of 28 ml, or 24 ml per 100 gm of live weight. During the next 42 days the mean consumption was 46 ml per day and 22 per 100 gm of live weight.

The water consumption of a growing rat increases only slightly as it grows and declines in relation to its total body weight. The consumption of sugar solutions increases as the rat grows and maintains a fairly constant ratio to the live weight.

In the course of these exploratory studies, other variables were introduced for comparison such as a commercial cola beverage as sold, a water solution containing H_3PO_4, (18 mg per liter) and a 10% sucrose solution containing this same

amount of H_3PO_4. All solutions containing 10% sucrose gave the same results so neither the phosphoric acid nor other constituents of the commercial cola beverage, such as caffeine, modified the amount drunk by the rat.

The effect of level of sugar in the diet upon the drinking of sugar solution

In feeding men in the Navy, the proposal had been made that less soft drinks would be purchased if more sugar were employed in the ration. Two diets were devised to determine if sugar in the diet might modify the rat's consumption of sugar solution. The percentage composition of diet A was as follows: sucrose 48, salt mixture 4, casein 25, dry brewer's yeast 10 (mixed 1/50 with irradiated yeast), hydrogenated cooking fat 10, and cod liver oil 3. Corn starch was substituted for sucrose in diet B.

Young adult rats were divided into 2 groups and caged individually for this study. Each had access to water and 10% sucrose solution. In the course of 4 weeks, (table 2) no difference was found between those fed the diet rich in sugar contrasted with those fed the starch diet. Very little water was drunk. The mean consumption of sucrose solution was 60 and 61 ml per day.

Using the same animals a second trial was run feeding diets still richer in carbohydrates. In this case the sugar or starch was mixed to comprise 74% of each diet while the per cent of all other constituents was cut in half. This was done by mixing equal weights of sugar with diet A, and starch with diet B. In table 3 these new diets are designated A_1 and B_1.

The results (table 3) provide no evidence that the sugar level of the diet modifies the amount of solution drunk by the rat. However, in the second experiment the quality of the diet was reduced in essential nutrients by dilution with carbohydrates. The amount of sucrose solution consumed by every individual on both the starch and sugar diets was reduced

TABLE 2

Rat experiment using high sugar and starch diets, and 10% sugar solution and water (7-12-44 to 8-8-44).

RAT NO.	FOOD CONSUMED		SUGAR SOLUTION CONSUMED		WATER CONSUMED		BODY WEIGHT	
	Total	Per day	Total	Per day	Total	Per day	Gained 7/12–8/8	On 8/8
	gm.	gm.	ml	ml	ml	ml	gm.	gm.
			Diet A (Sugar 48%)					
234	274.4	10	1956	70	56	2	63	406
235	344.7	12	1122	40	60	2	56	435
236	267.7	10	1926	69	31	1	66	439
237	266.4	10	1644	59	71	3	73	333
238	263.8	9	1816	65	62	2	83	411
239	233.4	8	1617	58	57	2	32	332
Aver.	275.1	10	1680	60	56	2	62	393
			Diet B (Starch 48%)					
240	315.5	11	1249	45	110	4	54	362
241	208.0	7	1920	69	79	3	30	372
242	264.2	9	2104	75	45	2	47	422
243	172.1	6	1795	64	15	1	..	364
244	207.1	7	1495	53	37	1	37	325
Aver.	233.4	8	1713	61	57	2	34	369

TABLE 3

Rat experiment using high sugar and starch diets (8-9-44 to 9-6-44).

RAT NO.	FOOD CONSUMED		SUGAR SOLUTION CONSUMED		VOLUME OF WATER CONSUMED		BODY WEIGHT	
	In 28 days	Per day	Total	Per day	Total	Per day	Gained	Final
	gm	gm	ml	ml	ml	ml	gm	gm
			Diet A_1 (Sugar 74%)					
234	336	12	1405	50	75	3	19	425
235	438	16	764	27	64	2	21	456
236	355	13	1264	45	56	2	13	452
237	295	11	894	32	39	1	1	334
238	257	9	1627	58	102	4	1	412
239	224	8	1232	44	65	2	—23	309
Aver.	318	11	1198	43	67	2	5	
			Diet B_1 (Starch 74%)					
240	449	16	677	24	101	4	30	392
241	275	10	1516	54	94	3	7	379
242	278	10	1802	64	74	3	11	433
243	291	10	1152	41	47	2	—2	362
244	231	8	1250	45	60	2	—3	322
Aver.	305	11	1279	46	75	3	9	

during this period of 4 weeks. This was the first of a number of observations that suggested the possibility of using the volume of sugar consumed by the rat as an indicator of deficiencies in diets.

Calcium in the diet and sucrose consumption

The experience with diets rich in carbohydrates indicated that the composition of the diet influenced the rat in the amount of sucrose beverage consumed, but did not indicate which of many variables might be responsible.

Additional clues were found in the course of another exploratory experiment. Plans were made to determine whether the teeth of growing rats were more subject to the etching effect of cola beverages when these rats were fed diets very low in calcium. A food mixture was prepared with the following percentage composition: corn meal 76, cottonseed oil 10, corn germ 5, dried brewer's yeast 5, cod liver oil 3, and sodium chloride 1. A second diet was prepared that would provide the animal with less calcium by mixing each 100 gm of the above feed with 50 mg of sodium oxalate.

Sixteen young rats were divided into 2 groups and placed upon the above diets. Each of these was subdivided so that 6 were given water to drink, 5 cola beverage and 5 of them 10% sucrose solution.

The original purpose of the experiment had to be abandoned because the consumption of the cola beverage and sugar solutions was so low that comparison could not be made with preceding studies. The average was 9 ml of sugar solution in contrast to 12 ml of water daily per 100 gm of live weight. In spite of the low calcium diets these rats all lived and doubled their body weights in 12 weeks.

In contrast a similar experiment was made with young hamsters during the same period and on the same diets. All of the hamsters died, indicating a much greater sensitivity of this species to low calcium diets.

At the end of 12 weeks, individuals were given supplements of bone meal or calcium carbonate or casein. There was some

response in each case as indicated by the drinking of more sucrose solution but this was the most marked in the case of those fed bone meal. Rats kept on the low calcium diet but changed from sugar solution to water responded at once by an increase in fluid consumption.

These exploratory studies indicated further that the amount of 10% sugar solution drunk by a rat represented a response to the quality of the diet. One of the factors indicated was calcium.

Response to calcium, vitamins and casein

Two years after the above observations were made at Bethesda, Maryland, this study was extended. External conditions were more uniform since the rats were now kept in an air-conditioned laboratory.

Two experiments were completed following the earlier method of diluting an adequate basal diet with starch. In this case the basal diet consisted of the stock feed used in rearing our breeding colony. In a preliminary trial with growing rats 3 parts of this basal diet were mixed with 1 of corn starch. In paired feeding trials young rats grew as well upon this diluted diet as upon the stock diet itself. Furthermore, the consumption of 10% sugar solutions by rats upon both diets was the same, indicating that rats sensed no deficiency in the basal diet.

Earlier experience had indicated this stock diet could be diluted even further without any reflection in the development of growing rats provided the diet were mixed with 3 parts of corn starch. Thus the protein was reduced to about 7%.

This mixture was used in 2 studies, 1 with adult rats 5 months of age, and 1 with rats 5 weeks old. In each case rats were paired on the basis of sex and litter. One rat of each pair was fed the stock diet: the other was fed the diet diluted with 3 parts of starch. Rats were caged individually and allowed all of the 10% sucrose solution they desired. The amount was measured daily using 100-ml graduated tubes.

In each study before adding supplements for test, a preliminary period of 8 weeks for the adult rats and 4 for the young ones was allowed until the differences between the consumption of sugar solutions by the 2 groups became significant.

After this, various supplements were added first singly, then in pairs and finally in trios. These additions included water-soluble vitamins, calcium gluconate, yeast, vitamin A, and casein.

The response could be judged by comparing the volume of solution drunk by the test animals and the controls. These supplements are indicated in tables 4 and 5.

The response of the older group to calcium in 3 different periods is evident (table 4). The consumption of the sugar solution in each case actually exceeded the control. In light of the recent findings (Kane and McCay, '47) that a higher level of calcium is required to maintain a rat in calcium balance as it grows older, this result may be partly explained. None of the other substances tested gave a response like calcium.

The results with the growing rats (table 5) were similar inasmuch as they responded to calcium but not when this was the sole addition. Neither calcium alone nor in combination with a water-soluble vitamin as riboflavin or a source of protein such as casein gave a response. However, when the supplement consisted of calcium, vitamin A and yeast or casein, the consumption of the sucrose solution exceeded the control.

Thus the growing rat would seem to sense a combined deficiency of calcium, vitamin A and protein but not one with respect to the water-soluble vitamins contained in the yeast. Inasmuch as this study was not made with synthetic diets known to be deficient in water-soluble vitamins, the only definite conclusion is that under these conditions the volume of sugar solution consumed may serve as a criterion of calcium deficiency and possibly of others such as protein.

Recent exploratory studies indicate that old rats in the last third of life may lose their abilities to sense some common deficiencies that are readily sensed in the first third of life.

TABLE 4

Consumption of sugar solution by young adult rats.

SUPPLEMENT PER DAY	TIME IN WEEKS	PAIRS NO. OF	AVERAGE ML DRUNK PER RAT BY PERIODS [1]		
			Experimental group	Control group	Significance
			Preliminary period		
			¾ basal plus ¼ starch	Basal	[2]
None	9	10	64.1 ± 19.0	59.1 ± 24.4	
			¾ basal plus ¼ starch	Basal	[2]
None	8	9	48.4 ± 19.7	66.9 ± 20.5	
			First supplementary period		
50 μg[3] thiamine	3	3	54.2 ± 18.4	84.8 ± 25.4	[2]
200 μg riboflavin	3	2	33.7 ± 7.2	67.4 ± 12.5	[2]
100 mg calcium	3	2	69.8 ± 28.3	69.0 ± 21.0	0
2.5 gm yeast	3	2	38.1 ± 10.8	63.5 ± 13.5	[2]
			Second supplementary period		
1.7 gm casein	5	3	54.2 ± 28.6	81.0 ± 30.6	[2]
1.7 gm casein + 6 μg vitamin A	5	3	58.7 ± 26.5	83.4 ± 9.5	[2]
1.7 gm casein + 6 μg vitamin A + 100 mg calcium	5	3	75.4 ± 29.2	60.7 ± 14.6	[2]
			Third supplementary period		
1.7 gm yeast	5	3	49.9 ± 16.9	80.3 ± 33.5	[2]
1.7 gm yeast + 6 μg vitamin A	5	3	67.9 ± 16.4	77.7 ± 7.8	[2]
1.7 gm yeast + 6 μg vitamin A + 100 mg calcium	5	3	94.1 ± 16.9	62.3 ± 8.8	[2]

[1] Means ± standard deviations.

[2] Significant at 1% level.

TABLE 5

Consumption of sugar solution by growing rats.

SUPPLEMENT PER DAY	TIME IN WEEKS	NO. OF PAIRS	AVERAGE ML DRUNK PER RAT BY PERIODS [1]		
			Experimental group	Control group	Significance
Preliminary period					
			¼ basal plus ¾ starch	Basal	[2]
None	4	10	17.6 ± 8.1	67.8 ± 30.0	
First supplementary period					
50 μg thiamine	5	3	18.5 ± 6.5	75.6 ± 8.7	[2]
200 μg riboflavin	5	3	17.5 ± 4.9	70.3 ± 27.0	[2]
100 mg calcium	5	2	22.4 ± 5.7	100.2 ± 13.0	[2]
2.5 gm yeast	5	2	23.8 ± 8.5	98.8 ± 22.2	[2]
Second supplementary period					
50 μg thiamine + 200 μg riboflavin	4	3	18.4 ± 4.9	79.3 ± 23.8	[2]
200 μg riboflavin + 100 mg calcium	4	3	22.5 ± 6.5	77.7 ± 26.3	[2]
1.2 gm casein + 100 mg calcium	3	2	36.0 ± 17.2	87.7 ± 4.3	[2]
1.2 gm casein + 50 μg thiamine	3	2	25.5 ± 8.3	88.7 ± 24.5	[2]
Third supplementary period					
1.2 gm casein	5	3	22.1 ± 11.9	79.7 ± 26.2	[2]
1.2 gm casein + 3 μg vitamin A	5	4	30.0 ± 19.8	85.6 ± 21.7	[2]
1.2 gm casein + 3 μg vitamin A + 100 mg calcium	5	3	86.7 ± 30.2	78.9 ± 18.9	0
Fourth supplementary period					
1.2 gm yeast	5	3	32.4 ± 6.4	70.0 ± 21.3	[2]
1.2 gm yeast + 3 μg vitamin A	5	4	44.4 ± 12.4	94.9 ± 16.2	[2]
1.2 gm yeast + 3 μg vitamin A + 100 mg calcium	5	3	98.3 ± 29.9	80.7 ± 14.9	[2]

[1] Means ± standard deviations.

[2] Significant at 1% level.

SUMMARY

Rats fed an adequate stock diet drink about 12 ml of water or 24 ml of 10% sucrose solution per day per 100 gm of live weight. The water consumption of growing rats in relation to body weight declines progressively as the rats mature, while the sugar solution drunk is relatively constant.

No evidence has been found that rats tire of a sugar solution. Diets rich in sugar or starch produce no decrease in the drinking of sucrose solutions unless the high carbohydrate dilutes essential nutrients to a low level.

When fed diets low in calcium rats refuse to consume cola beverage or sucrose solution except in amounts to provide water. Under such conditions the amount of sucrose solution drunk is lower than normal water consumption.

When an adequate stock diet was diluted with 3 parts of corn starch, the amount of sucrose solution consumed was observed to depend upon supplementing the diet with calcium in the case of adult rats; in the case of growing rats, a high level of sucrose solution was drunk only when the diet was supplemented with protein, vitamin A and calcium.

An adult rat drinking daily 72 ml of sucrose solution excreted 43 ml of urine in contrast to a rat drinking 40 ml of water and excreting 15 ml of urine.

Rats lactating heavily drank 69–71 ml of sucrose solution and 17–24 ml of water.

Exploratory studies indicate that the rat's ability to detect certain dietary deficiencies may decrease with advancing age.

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PROTEIN ASSAY BY RAT GROWTH:

A COMPARISON OF (A) LITTER MATES VS. RANDOMLY SELECTED MALES, AND (B) MODERATE RESTRICTION OF FOOD INTAKE VS. AD LIBITUM FEEDING

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ONE FIGURE

(Received for publication June 13, 1947)

Numerous laboratories have adopted rat growth methods as means for an evaluation of proteins and protein hydrolysates. Direct comparison of results among several laboratories shows striking qualitative agreement; quantitative variations are encountered as direct sequelae of a host of variables. To name only a few these would include ration composition, animal strain, level of test feeding and caloric intake of animals.

Studies of the roles of some of these variables under homogeneous conditions have been reported from many laboratories. Mitchell ('44) and Barnes et al. ('45) have recently compared *ad libitum* and paired feeding technics. Barnes and Bosshardt ('46) have demonstrated the effects of level of protein fed on rat growth and protein efficiency. Forbes et al. ('46) showed the consequences of variation of fat level in the ration on rat growth. Vitamin supplement effects have been explored by Bosshardt et al. ('45), Harte and Travers ('47), Harris and Hove ('47) and others. Whether strepogenin plays a role in protein utilization has been explored by Woolley ('45) and Womack and Rose ('46). Comprehensive bibliographies are given by Block and Mitchell ('46–'47) and Mitchell and Hamilton ('29).

In the establishment of a standard method for evaluating a protein or protein hydrolyzate for pharmacopoeial purposes, for example, certain requirements must be met. The technic decided upon must be relatively simple, yet reproducible; it should give uniform results in the hands of any laboratory group engaged in such work and must have good discriminatory capacity. For both control and enforcement purposes the test devised must not permit making too many errors of either the first or second kind; namely, rejection of satisfactory preparations or acceptance of sub-standard preparations.

This report deals with a survey of 2 characteristics of experimental design which might be expected to play a role in the discriminatory capacity of such a test, namely, (1) the use of litter mates and (2) the restriction of dietary intake imposed by putting an upper limit on the amount of food offered. Data on roles played by these factors do not seem to be available in the literature. The imposition of an upper limit on amount of food offered has been successfully used in protein regeneration studies conducted on the rat by Frazier et al. ('47). In the study reported in the present paper a total of 99 animals were observed when fed an identical casein ration and the data subjected to statistical analysis.

EXPERIMENTAL

Table 1 shows the ration used in these experiments. This diet is estimated to provide 4.5 cal. per gm. Ten litters (6 rats each) and 50 randomly picked males [1] were caged separately in an air-conditioned animal room (76°F.) and put on our basic casein ration. After 1 week 2 animals had died and 1 had failed to gain more than 5 gm and was therefore discarded.

At this time the remaining litter mates were divided into 5 groups of 10 to which 3 and 2 represented matched units with respect to litter, sex and average weight (see table 2).

[1] From Rockland Farms. The cooperation of Harry G. Herrlein of Rockland Farms in making these litters and the randomly selected males available is greatly appreciated.

The surviving 49 randomly selected males were divided into 4 groups of 10, and 1 of 9, adjusted only to give corresponding average weights. These groups were designated A′, B′, C′, D′, and E′ and the 10 groups were maintained on the casein ration according to the schedule, groups A, B, C and A′, B′, C′

TABLE 1

Composition of ration used.

Lard	90.0 gm	Thiamine	5 mg
Salts[1]	40.0 gm	Riboflavin	8 mg
Cod liver oil	20.0 gm	Niacin	13 mg
Wheat germ oil	10.0 gm	Calcium pantothenate	13 mg
Choline	1.8 gm	Pyridoxine	6 mg

Casein[2] to yield 1.6% nitrogen
Starch q.s. to make a total of 1 kg

[1] GBI (General Biochemicals, Inc.) salt mixture was used.
[2] Labco Vitamin Free casein (Borden) was used.

TABLE 2

Distribution of groups.

LITTER MATES				RANDOMLY SELECTED MALES	
Group	Distribution by sex		Average body weight	Group	Average body weight
			gm		gm
A	4 ♂	6 ♀	73.2 ± 3.9[1]	A′	78.3 ± 2.2
B	4 ♂	6 ♀	73.7 ± 1.7	B′	78.5 ± 3.4
C	4 ♂	6 ♀	73.2 ± 1.7	C′	77.9 ± 2.7
D	6 ♂	4 ♀	75.0 ± 1.8	D′	72.1 ± 3.1
E	6 ♂	4 ♀	75.2 ± 3.2	E′	74.0 ± 2.4

[1] Indicated limits represent standard error (s.e.) of the mean, where

$$\text{s.e.} = \sqrt{\frac{(X-\bar{x})^2}{n\,(n-1)}}\,;\ X \text{ being}$$

individual values and $\bar{x}$ the group mean, n representing the number of individuals. This convention is employed in all following tables, where relevant.

being restricted to not more than 10 gm of ration daily; groups D, E, and D′, E′ were fed *ad libitum*. Water consumption was not restricted for either group. Food intakes were measured and spillage was collected daily. The animals were weighed weekly.

RESULTS

During the first week 2 rats died. The deaths occurred in the litter groups C and E and were of animals that had gained 9 and 16 gm, respectively, during the pre-conditioning period. During this first week, too, 3 animals gained less than 5 gm, although they had respectively gained 8, 22 and 25 gm previously. One additional fatality occurred in group B′ during the third week of the test period.

At the end of 4 weeks the weight gains were scored and analyzed. The results will be considered, first, for those animals restricted to 10 gm of ration daily. Table 3 gives the weight gains, and protein efficiencies, together with their

TABLE 3

Weight gains and protein efficiencies of groups with restricted food intake.

GROUP	N	WEIGHT GAIN	PROTEIN EFFICIENCY	REMARKS
		gm		
A	10	60.9 ± 2.0	2.39 ± 0.08	Litter mates Groups
B	10	62.2 ± 1.4	2.35 ± 0.05	
C	9	63.3 ± 1.7	2.37 ± 0.06	
A′	10	60.0 ± 2.8	2.42 ± 0.08	Groups of randomly selected males
B′	9	62.1 ± 1.8	2.40 ± 0.06	
C′	10	62.9 ± 2.1	2.38 ± 0.07	

standard errors. From this table it is evident that there is no significant difference between any 2 of the 6 groups, whence there appears to be no advantage in using litter mates as compared to randomly chosen males.

Since 1 of the litter mates in group C had died during the first week, the data for its siblings were segregated from the other 2 groups and an analysis of variance carried out by standard methods as described by Fisher ('41) and Snedecor ('40). This analysis showed no reduction in mean square value among litter mates.

The data coming from the groups fed *ad libitum* (table 4) showed strikingly different results. The mean gains were somewhat greater and more irregular, with the concomitant

occurrence of much greater standard errors. Protein efficiencies were not very different for both random males groups, but the values for the litter mates groups do not agree well.

When the weight data for the sibling of the rat in group E which died were discarded from group D and an analysis of variance carried out, the difference between litter mates is, surprisingly, statistically significant, with an associated probability of about 0.03. Similar examination of the corresponding protein efficiency data also reveals a significant statistical difference, with an associated probability of about 0.01.

TABLE 4

Weight gains and protein efficiencies of groups fed ad libitum.

GROUP	n	WEIGHT GAIN	PROTEIN EFFICIENCY	REMARKS
		gm		
D	10	69.7 ± 4.3	2.18 ± 0.08	Litter mates
E	9	82.3 ± 4.7	2.45 ± 0.06	Groups
D′	10	81.6 ± 7.4	2.44 ± 0.10	Groups of randomly selected males
E′	9	75.1 ± 2.3	2.36 ± 0.06	

Of some interest, too, is the fact that the spillage average for the 58 rats whose rations were restricted was only 8.8 gm during the 28-day period, while the 38 animals fed *ad libitum* apparently lacked the incentive of restriction to acquire neater eating habits and showed an average spillage of 39.5 gm.

At the end of 6 weeks on these rations the data were again analyzed; the relative results were entirely unchanged and therefore are not reported in detail.

DISCUSSION

Since the basic experimental design was such as to exclude the existence of any differences between rations the statistical conclusions apply to situations apt to be encountered more-or-less rarely in assays where no true differences exist between rations being compared. We feel that these data suggest that the added refinement of the litter mate technic offers

no advantage under conditions where randomly grouped males from single shipments of not less than 50 animals from a reasonably homogeneous source of supply are used.

The sole advantage which might be expected to accrue from the use of litter mates when differing rations are undergoing comparison is greater homogeneity of the variance in groups so selected. Examination of the data in table 3 makes the application of the appropriate statistical test (Snedecor, '40) superfluous. It is obvious that the variances for groups A, B, and C and for groups A′, B′ and C′ are equal and equally homogeneous.

On the other hand, the data in table 4 suggest that the variances associated with the growth responses in the 2 groups of randomly selected males may not be homogeneous; application of Bartlett's test gives a value for chi squared which just borders on conventional statistical significance.

In experiments such as those reported here one might postulate that if siblings of the same sex have greater similarity of growth potential than randomly chosen animals, then the variance within litters ought to be less than the variance between litters. Not only is this expectation not realized, but with the *ad libitum* fed animals the contrary result definitely emerges. Within the framework of these data the postulate is not supported and doubt is cast on the premise from which it arises. Of course these conclusions rest on data yielded by only 45 animals and definitive affirmation of the thesis must await experimentation on a larger scale.

The pitfalls in experiments of this sort are well exemplified by the results obtained with groups D and E. Unless one had *a priori* reasons to expect no difference, such a result would pass unchallenged in a routine test and the ration fed the animals in group D would be adjudged inferior whether the data were merely compared in a rough fashion or subjected to scrupulous statistical analysis.

There seems, however, to be little question that material improvement in precision results from the moderate degree of food intake restriction imposed in part of this experiment. For the whole experiment, the effect of restriction was to

diminish by almost 20% the food consumption from the *ad libitum* level.

It is most important to reduce the experimental variance as much as possible to help avoid making the errors, first, of reporting differences where none exist, and second, of failing to recognize differences which do exist. For example, previous experiments have suggested that randomly chosen males fed 10% casein *ad libitum* in somewhat similar rations gain on the average 80 gm in a 30-day period, with a variance, for a single animal, of 400 $\overline{\text{gm}}$.[2] (The pooled variance for groups D′ and E′ is 316 $\overline{\text{gm}}^2$; the pooled mean, 78.5 gm.) When these were assumed to be the true parameters of the performance of casein it was possible to calculate for all experimental preparations whose true mean gain values ranged from 50 to 100 gm with constant variance (400 $\overline{\text{gm}}^2$) the probability of making false decisions on a 90% criterion; i.e., preparations whose performance is poorer than 90% of the performance of casein are to be rejected.

When these estimations were made it appeared that a false decision would be made on the average 3 times in every 15 assays of products whose true values were in the range from 60–120% of a standard; further, 2 of these false decisions would involve acceptance of preparations poorer than 90% of casein while 1 would involve rejection of a preparation better than 90% of casein.[2]

With temporary acceptance of the parameters established by this study for animals fed casein on restricted intake (mean gain = 62 gm, variance = 40 $\overline{\text{gm}}^2$), the situation with respect to the probability of false decisions is substantially improved. Under these circumstances the likelihood of false decision is reduced to 1 in about 16 assays (with respect to the same range of products), and of every 12 false decisions 5 will be false rejections and 7 false acceptances, the 90% criterion being retained. The calculations are summarized in figure 1.

[2] Dr. William J. Youden has been kind enough to review our calculations and confirm these statements. His friendly interest is gratefully acknowledged.

The benefits thus accruing for assay purposes are substantial and may outweigh the physiological objections that moderate dietary restriction might justify.

However, these are purely mathematical considerations, pessimistic to the extent that they do not take into account the correlation which exists between the growth responses of standard and test material subjected to parallel replication.

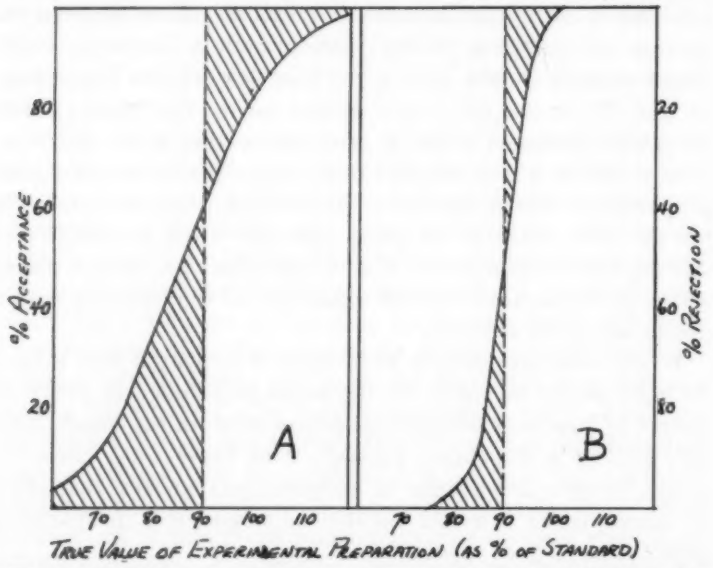

Fig. 1 Probability of acceptance and rejection of experimental preparations referred to a 90% of standard criterion. A — *ad libitum* fed animals ($n = 10$; assumed parameters — $M = 80$ gm, $\sigma^2 = 400$ $\overline{\text{gm}}^2$). B — Animals fed with moderate restriction ($n = 10$; assumed parameters — $M = 62$ gm, $\sigma^2 = 40$ $\overline{\text{gm}}^2$). Cross hatched areas represent regions of false decision.

Before a final decision can be reached it will be necessary to explore more fully the physiological aspects of the restrictive technic. Experiments with this in view are in progress.

SUMMARY

1. Groups of 9 or 10 randomly chosen male albino weanling rats, selected from groups of 50 or more, give mean growth

response (and variance) of precisely the same order of magnitude as groups from the same stock which are paired with respect to sex and litter.

2. Preconditioning of the experimental population by feeding for 1 week on a 10% casein ration offers some improvement but does not entirely eliminate fatalities or irregular performance during subsequent weeks on test.

3. Partial restriction of food intake (to not more than 10 gm daily) reduces the mean growth response over 28 days on 10% casein rations by approximately 20% from the mean growth of *ad libitum* fed animals.

4. The variance for restricted fed animals is only about one-eighth to one-tenth that observed for *ad libitum* fed animals.

5. The reduction of variance offers approximately 3.5-fold improvement in the discriminatory capacity of an assay test set-up on a 90% criterion.

6. The physiological implications of the partial food restriction technic remain to be elucidated.

ACKNOWLEDGMENTS

The assistance of Dorothy Nicholson and Louis Quesal in this work is greatly appreciated.

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THE COMPARATIVE BIOLOGICAL AVAILABILITIES OF FERROUS SULFATE IRON AND FERRIC ORTHOPHOSPHATE IRON IN ENRICHED BREAD[1]

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THREE FIGURES

(Received for publication May 17, 1947)

According to the flour and bread enrichment program, as developed by federal and state agencies, the required iron should be added to the flour only in forms which are harmless and assimilable (Fed. Reg., '41). Primary consideration has naturally been given to the selection of iron preparations which exert no deleterious action upon the flour or bread. This technical requirement has somewhat obscured the basic nutritional motive for enrichment in that the degree of biological availability of the added iron has not been thoroughly considered. As has been pointed out by the Council on Foods and Nutrition of the American Medical Association ('41), obviously there is no nutritional advantage in adding an iron compound if this iron is not satisfactorily available to the body. Tobey and Cathcart ('41) pointed out that little information had been reported on the assimilability of iron compounds that were already in use for enrichment of flour.

Several forms of iron have been of practical interest for enrichment. The initial report on ferric phytate gave a somewhat favorable impression (Andrews, Evans and Huber, '41),

[1] Presented in part before the American Institute of Nutrition, Chicago, May 21, 1947 (Blumberg, H., and A. Arnold, '47, Fed. Proc., *6*: 402).

but in subsequent rat tests (Nakamura and Mitchell, '43) and human studies (Moore, Minnich and Dubach, '43) it was found to be a comparatively poor source of iron. Little or no ferric phytate is now being used for flour enrichment. Sodium iron (ferric) pyrophosphate ($Fe_4(P_2O_7)_3 \cdot 2Na_4P_2O_7 \cdot 6H_2O$) was at first reported to be well utilized (Nakamura and Mitchell, '43), but in later investigations it was reported to have low availability (Street, '43; Freeman and Burrill, '45; Blumberg and Arnold, '47). Except for special products, sodium iron pyrophosphate is no longer being used to a great extent for enrichment of flour and bread. Reduced iron has been found to be highly available in rat investigations (Nakamura and Mitchell, '43; Freeman and Burrill, '45; Blumberg and Arnold, '47), as well as efficacious in clinical therapy (Fowler and Barer, '39). Reduced iron is now widely used in the mill enrichment of flour, as well as in other dietary products, such as infant foods and yeast.

Ferric orthophosphate has been the form of iron generally used when enrichment has been effected at the bakery. Day and Stein ('38) found ferric orthophosphate to be much less effective than ferric chloride for hemoglobin formation in rats. In their clinical studies on iron absorption, Moore and coworkers ('39) reported that the relatively insoluble ferric orthophosphate, as well as ferrous phosphate, was very poorly absorbed as compared with ferrous sulfate. In a single experiment on rats, Freeman and Burrill ('45) ranked ferric orthophosphate as only slightly less effective than the highly available ferric chloride. However, in 2 single-level experiments conducted as part of a comparative survey of iron sources, Blumberg and Arnold ('47) observed ferric orthophosphate to be less than one-half as effective as ferrous sulfate when the compounds were fed in enriched breads.

Ferrous sulfate is well known both experimentally and clinically as one of the most highly available forms of iron (Goodman and Gilman, '41). Recently ferrous sulfate has become of interest for bread enrichment.

In view of the large extent to which enrichment of the nation's bread is conducted at the bakery, it was considered desirable to obtain a better quantitative comparison of the relative biological availabilities of ferrous sulfate and ferric orthophosphate by testing each form of iron at several levels. The compounds were fed in the form of enriched breads containing the different iron sources, so that the iron would be tested in the same form as used for human consumption. A secondary comparison with ferric chloride was also made because some investigators have used the latter compound rather than ferrous sulfate as a standard of high biological availability.

EXPERIMENTAL METHODS

Diets

The diet employed in these experiments was similar to that previously used (Blumberg and Arnold, '47); the composition is given in table 1. Casein was included in the diet as a supplement to the inadequate protein of the bread, so as to provide sufficient protein for optimal hemoglobin regeneration. Low-iron casein, containing approximately 15 μg of iron per gm, was prepared in the laboratory from skimmed milk. The low-iron salt mixture was prepared by modifying U.S.P. XI Salt Mixture no. 2 in the following manner. The ferric citrate was omitted, of course. Since the bread supplied sodium chloride, this also was omitted from the mixture and the amount of salt mixture used was reduced from the usual 4% of the diet to 3%. Furthermore, potassium biphosphate was substituted for sodium phosphate.

Four lots of bread were baked from the same lot of flour with special enrichment mixes that supplied the usual amounts of thiamine, riboflavin, and niacin, but varied with respect to iron. One lot of bread was for the negative control and contained no added iron. The other 3 lots were enriched to provide approximately the following quantities of iron: (1) 131 μg/gm, as ferric orthophosphate; (2) 42 μg/gm, as ferrous sulfate; and (3) 21 μg/gm, as ferric chloride. The ferric orthophosphate was from a batch actually used for commercial

enrichment; the exsiccated ferrous sulfate was U.S.P. grade, and the ferric chloride was C.P. grade.

The breads were air-dried at 37°C. to a moisture content of approximately 4% and were then ground for use in the diets. Iron analyses of the breads were made by a thiocyanate procedure (Eckert and Auerbach, '44). The iron content of the negative control bread was found to be about 12 μg/gm. The actual analyses indicated that the supplemented breads had slightly more than the intended additional iron contents, as follows: ferric orthophosphate 132 μg/gm, ferrous sulfate

TABLE 1

Composition of diet.

BASAL MIXTURE		SUPPLEMENTS PER 100 GM BASAL MIXTURE [1]	
	%		*mg*
Bread (dried)	82	Thiamine hydrochloride	1
Casein (low-iron)	12	Riboflavin	2
Salt mixture (low-iron)	3	Pyridoxine hydrochloride	1
Corn oil	3	Calcium pantothenate	4
		Niacinamide (nicotinamide)	2
		Choline chloride	100
		Inositol	100
		Copper (as $CUSO_4 \cdot 5H_2O$)	3
		Manganese (as $MnSO_4 \cdot H_2O$)	1.5

[1] Each rat received by stomach tube a weekly fat-soluble vitamin supplement equivalent to 2000 U.S.P. units of vitamin A, 400 U.S.P. units of calciferol (vitamin D_3), and 10 mg of alpha-tocopherol.

43 μg/gm, and ferric chloride 26 μg/gm. By suitable dilution with the negative control bread, the iron-enriched breads were made into the series of test bread mixtures shown in table 2. The 4 levels of ferric orthophosphate iron increased in geometric progression by multiples of 2.5, as follows: 8.4, 21.0, 52.5, and 131.2 μg/gm of bread, or 6.9, 17.2, 43.1, and 107.6 μg/gm of diet. The 4 levels of ferrous sulfate iron increased by multiples of 2, as follows: 5.25, 10.5, 21.0, and 42.0 μg/gm of bread, or 4.3, 8.6, 17.2, and 34.4 μg/gm of diet. The 2 levels of added ferric chloride iron were made the same as the intermediate levels of ferrous sulfate, i.e., 10.5 and 21.0 μg/gm of

bread or 8.6 and 17.2 μg/gm of diet, in order to permit comparison of these 2 highly available iron preparations.

The iron contents of the various diets are given in table 2. The extraneous, non-bread iron in the diets was only about 2 μg/gm. Diet 12 was prepared by addition of ferrous sulfate to the negative control diet 1 at a level of 244 μg of iron per gm of bread, or 200 μg/gm of diet. This provided a positive control to demonstrate the maximum rate of hemoglobin regeneration permitted by the basal diet in the presence of an amount of available iron known to be well within the optimal range.

TABLE 2

Iron contents of breads and diets.

GROUP	BREAD		NO. RATS	IRON CONTENT OF BREAD	IRON CONTENT OF DIET
	Compound added	Iron added			
		μg/gm		μg/gm	μg/gm
1	None (negative control)	...	9	12.0	11.9
2	Ferric orthophosphate	8.4	8	20.4	18.8
3	Ferric orthophosphate	21.0	8	33.0	29.2
4	Ferric orthophosphate	52.5	9	64.5	54.9
5	Ferric orthophosphate	131.2	10	143.0	118.0
6	Ferrous sulfate	5.25	9	17.2	16.2
7	Ferrous sulfate	10.5	9	22.5	20.5
8	Ferrous sulfate	21.0	10	33.0	29.2
9	Ferrous sulfate	42.0	9	54.0	45.0
10	Ferric chloride	10.5	9	22.5	20.5
11	Ferric chloride	21.0	10	33.0	29.2
12	Ferrous sulfate (positive control)	244.0	7	256.0	212.0

Animal experimentation

Albino rats of the Sherman strain were prepared for iron-deficiency studies by special feeding precautions generally similar to those described by Elvehjem and Kemmerer ('31). At about 25 days of age the weanling rats were removed to individual galvanized cages in which there was no exposed iron or rust. Anemia was induced by feeding certified cow's milk supplemented with cupric sulfate and manganous sulfate equivalent to 1.8 mg of copper and 1.4 mg of manganese

per liter. After the rats had been on the iron-depletion diet for 35 days, hemoglobin determinations were made on tail blood by the alkaline hematin method, as adapted for the Klett-Summerson colorimeter. Except for 12 somewhat resistant animals, the rats were found to be sufficiently anemic for test purposes, i.e., the hemoglobin values were 2.5–5 gm/100 ml, with an average of about 3.9 gm/100 ml.

The animals were divided into groups of 10 rats each, except for the positive control group 12, which had only 7 rats. Weight and sex distributions were similar in the various groups. Occasional mortality during the experiment reduced the numbers slightly, so that the final test groups contained 8–10 rats each, as shown in table 2. The experimental diets were then fed for 4 weeks, hemoglobin determinations and weighings being made at the end of each week. Groups 1, 2, 3, and 6, which were regenerating hemoglobin at slow rates, were continued on experiment for as long as 9 weeks for determination of the length of time required to reach a hemoglobin value of 10 gm/100 ml, i.e., close to the beginning of the normal range.

RESULTS

Ferrous sulfate and ferric orthophosphate

The general nature of the results of the comparison between ferrous sulfate and ferric orthophosphate is shown by the hemoglobin curves in figure 1. The rats fed the negative control diet and the 2 lower levels of ferric orthophosphate had not shown any increase in their hemoglobin concentration values by the end of the first week. However, since the values did increase during subsequent weeks, the readings at 1 week did not appear to offer a suitable basis for a valid comparison. On the other hand, some of the animals in the faster regenerating groups had already reached the normal range of hemoglobin values by the end of the second week. Consequently, the interpolated value for 1.5 weeks appeared to be the most sensitive point for comparison. When some later point on the curve is used, the quantitative superiority of the more highly

available forms may not be so marked or indeed may no longer be evident. With minor exceptions, the general trend of the results at 1.5 weeks is confirmed by the curves for other points during the test. The marked quantitative superiority of ferrous sulfate iron over ferric orthophosphate iron is readily apparent at the various levels of enrichment.

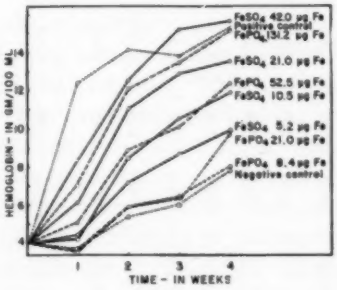

Figure 1

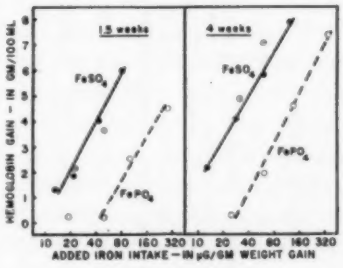

Figure 2

Fig. 1 Hemoglobin regeneration curves of anemic rats fed the indicated levels of ferrous sulfate iron (———) or ferric orthophosphate iron (– – – –), as supplied by enriched bread. Negative and positive control curves are also shown. All points were corrected to an initial hemoglobin value of 4.0 gm/100 ml of blood.

Fig. 2 Dosage response curves at 1.5 weeks and at 4 weeks of anemic rats fed ferrous sulfate iron or ferric orthophosphate iron, as supplied by enriched bread. The ferric chloride points are shown by the circled dots.

The initial hemoglobin values, as listed in table 3, show that the various test groups were at approximately the same degree of anemia at the start of the experiment. The mean hemoglobin gains and iron intakes after 1.5 weeks and 4 weeks are also given in table 3. These data were used to arrive at a definite figure for the comparative availability of ferrous sulfate and ferric orthophosphate in the following way. The total hemoglobin gains were corrected for the hemoglobin gain of the negative control (group 1), and the total iron intakes were corrected for the iron intake due to the diet itself without added iron (11.9 µg/gm). This permitted calculation of hemoglobin gain per µg of added iron intake. The added iron intakes were also corrected for the minor differences in

TABLE 3

Responses of rats to various sources and levels of iron.

GROUP	BREAD		NO. RATS	INITIAL WT. (AV.)	WT. GAIN (AV.)		FOOD CONSUMPTION (AV.)		IRON INTAKE (AV.)		INITIAL HEMOGLOBIN (AV.)	HEMOGLOBIN GAIN MEAN ± S.E.	
	Compound added	Iron added			1.5 wks.	4 wks.	1.5 wks.	4 wks.	1.5 wks.	4 wks.		1.5 wks.	4 wks.
		µg/gm		*gm*	*gm*	*gm*	*gm/day*	*gm/day*	*µg/day*	*µg/day*	*gm/100 ml*	*gm/100 ml*	*gm/100 ml*
1	None (negative control)	...	9	96	35	94	9.8	11.3	117	134	3.93	0.48 ± 0.26	3.69 ± 0.33
2	Ferric orthophosphate	8.4	8	84	36	90	9.4	12.3	177	231	3.90	0.69 ± 0.32	3.99 ± 0.44
3	Ferric orthophosphate	21.0	8	100	34	90	9.1	11.4	266	333	4.01	0.68 ± 0.33	5.64 ± 0.64
4	Ferric orthophosphate	52.5	9	92	45	111	10.5	12.9	576	708	3.86	2.96 ± 0.51	8.31 ± 0.57
5	Ferric orthophosphate	131.2	10	98	46	106	11.8	12.9	1389	1518	3.88	4.99 ± 0.76	11.11 ± 0.41
6	Ferrous sulfate	5.25	9	94	39	104	10.9	11.7	176	190	3.87	1.79 ± 0.14	5.84 ± 0.22
7	Ferrous sulfate	10.5	9	101	46	106	11.3	12.2	232	250	3.99	2.30 ± 0.37	7.82 ± 0.63
8	Ferrous sulfate	21.0	10	84	44	100	10.5	12.2	307	356	3.57	4.57 ± 0.39	9.48 ± 0.63
9	Ferrous sulfate	42.0	9	93	46	96	11.0	12.9	495	580	3.81	6.43 ± 0.28	11.62 ± 0.51
10	Ferric chloride	10.5	9	93	38	85	9.2	11.1	188	228	4.17	2.64 ± 0.45	8.62 ± 0.83
11	Ferric chloride	21.0	10	80	36	89	9.5	11.3	277	330	3.85	4.09 ± 0.29	10.77 ± 0.34
12	Ferrous sulfate (positive control)	244.0	7	84	45	101	8.7	11.1	1843	2352	3.39	9.23 ± 0.75	11.53 ± 1.8

the average weight gains of the groups, although such correction did not modify the final conclusions. This permitted calculation of hemoglobin gain per μg of added iron intake per gm of weight gain. The resultant dosage response values, illustrated graphically in figure 2, were then compared statistically by the method of Waddell and Kennedy ('47).

At 1.5 weeks ferric orthophosphate iron was 19.6 ($\pm$ 2.4)% (mean $\pm$ S.E.) as available as ferrous sulfate iron if the lowest ferric orthophosphate value (group 2) is omitted from the calculation. This may be done on the grounds that the latter point appears to be below the sensitive portion of the dosage response curve. However, comparison of the 2 iron sources without omitting the group 2 value does not change the result markedly, though the standard error does not give so true a picture of the agreement of the data. Calculated to include all points at 1.5 weeks, ferric orthophosphate iron was 21.2 ($\pm$ 6.8)% as available as ferrous sulfate iron.

At 4 weeks ferric orthophosphate iron was 25.2 ($\pm$ 2.0)% as available as ferrous sulfate iron. As indicated previously, the latter time does not really give a valid comparison, but it has been included to demonstrate the marked differences in availability which exist even at this less sensitive point.

The excellent agreement between the groups fed differing amounts of the same added iron source is demonstrated in figure 2. Aside from the 1.5-week value for group 2, as noted above, the points fall very nearly on straight lines.

The statistical significance of the differences between the individual groups was determined by calculation of the standard error of the difference between the mean hemoglobin gains both at 1.5 weeks and at 4 weeks, comparisons being made for all groups. A relatively high criterion of significance was adopted by basing conclusions only on P values of 0.01 or less (i.e., probability of the difference being fortuitous equals 1 in 100, or less). The results of this analysis showed that the previously mentioned differences in mean hemoglobin gains between the ferric orthophosphate and ferrous sulfate groups were highly significant. For instance, at 1.5 weeks group 7

($FeSO_4$ iron 10.5 μg/gm) was significantly superior ($P = 0.005$) to group 3 ($FePO_4$ iron 21 μg/gm), and was not significantly different ($P = 0.3$) from group 4 ($FePO_4$ iron 52.5 μg/gm). Likewise, group 9 ($FeSO_4$ iron 42 μg/gm) was greatly superior ($P = < 0.001$) to group 4 ($FePO_4$ iron 52.5 μg/gm), although not significantly superior ($P = 0.09$) to group 5 ($FePO_4$ iron 131.2 μg/gm). The results of the analysis at 4 weeks were almost as significant.

TABLE 4

Rates of hemoglobin regeneration, based upon days required to reach hemoglobin value of 10 gm per 100 ml.

GROUP	BREAD		DAYS	RATE OF HEMOGLOBIN REGENERATION AS PERCENTAGE OF OPTIMUM
	Compound added	Iron added		
		μg/gm		
1	None (negative control)	...	63.0	0
2	Ferric orthophosphate	8.4	47.4	3
3	Ferric orthophosphate	21.0	37.8	6
4	Ferric orthophosphate	52.5	20.5	18
5	Ferric orthophosphate	131.2	10.7	42
6	Ferrous sulfate	5.25	28.7	11
7	Ferrous sulfate	10.5	18.9	21
8	Ferrous sulfate	21.0	12.4	36
9	Ferrous sulfate	42.0	9.8	47
10	Ferric chloride	10.5	20.1	19
11	Ferric chloride	21.0	13.3	33
12	Ferrous sulfate (positive control)	244.0	5.4	100

The results were also calculated in terms of the number of days required to reach an average hemoglobin value of 10 gm/100 ml, which is close to the normal range (see table 4). The various groups were then rated for percentage of optimal hemoglobin regeneration by comparison with the positive control, group 12, which contained a known excess of ferrous sulfate. A correction was made for the hemoglobin regeneration contributed by the iron present in the diet without iron enrichment (group 1, negative control bread). By this method of calculation, also, it was demonstrated that the ferrous sul-

fate iron was approximately 4 to 6 times as effective as ferric orthophosphate iron. Statistical treatment of these data by the previously mentioned procedure (Waddell and Kennedy, '47) showed ferric orthophosphate iron to be 17.2 ($\pm$ 6.2)% as available as ferrous sulfate iron. This is in general agreement with the comparison based on hemoglobin regeneration at 1.5 weeks.

As shown in table 3, all of the groups of rats grew well. The weight gains of the various groups differed little and thus were in marked contrast to the wide variations in hemoglobin regeneration. Likewise, the differences in food consumption were small, corresponding generally to the small differences in weight gains, and could not account for the large differences in hemoglobin regeneration. In addition, several ferrous sulfate rats were pair-fed with ferric orthophosphate rats to maintain the same individual food consumption. The ferrous sulfate animals again proved much superior in hemoglobin regeneration. A comparison of iron intakes and hemoglobin gains (see table 3) emphasizes the superiority of the biological availability of ferrous sulfate iron over that of ferric orthophosphate iron.

Ferrous sulfate and ferric chloride

The comparison of ferrous sulfate and ferric chloride at 2 levels showed these forms of iron to be equally effective for regeneration of hemoglobin. The curves for the 2 compounds are practically superimposable, as may be seen in figure 3. Further evidence of similarity is presented by the hemoglobin gains in table 3 and the hemoglobin regeneration rates in table 4. Calculations were made for the standard error of the difference between the mean hemoglobin gains. The analysis at 1.5 weeks showed that there was no significant difference ($P = 0.56$) between group 7 ($FeSO_4$ iron 10.5 µg/gm) and group 10 ($FeCl_3$ iron 10.5 µg/gm). Similarly, there was no significant difference ($P = 0.32$) between group 8 ($FeSO_4$ iron 21 µg/gm) and group 11 ($FeCl_3$ iron 21 µg/gm). The analysis at 4 weeks likewise demonstrated that there was no significant

difference between the ferrous sulfate and ferric chloride values. The highly available ferric chloride iron, like that of ferrous sulfate, was 4 to 5 times as effective as ferric orthophosphate iron (see fig. 2). This study indicated that results based on ferric chloride standards should be directly comparable with those based on ferrous sulfate standards.

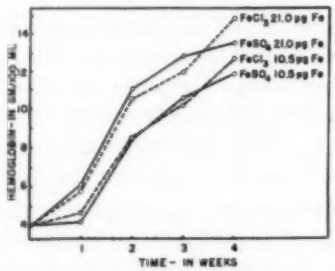

Fig. 3 Hemoglobin regeneration curves of anemic rats fed similar levels of ferrous sulfate iron (———) and ferric chloride iron (– – – –), as supplied by enriched bread. All points were corrected to an initial hemoglobin value of 4.0 gm/100 ml of blood.

DISCUSSION

Several experiments have now been reported on the utilization of ferric orthophosphate iron in rats. Although Day and Stein ('38) did not attempt a truly quantitative comparison, their results in a single-level study indicated that ferric chloride iron was approximately 4 times as available as ferric orthophosphate iron. In their single experiment, Freeman and Burrill ('45) found ferric orthophosphate iron to be practically as effective as ferric chloride iron. Inasmuch as Freeman and Burrill report only the final hemoglobin values at the end of 28 days, a time at which the groups were already well within the normal hemoglobin range, it is possible that differences occurring at 1 to 2 weeks were no longer apparent. In 2 previous single-level experiments, Blumberg and Arnold ('47) found ferrous sulfate iron to be more than twice as available as ferric orthophosphate iron. The present investigation at multiple levels appears to leave little doubt that, under the

conditions of these experiments, ferrous sulfate iron or ferric chloride iron is approximately 4 to 5 times as available for hemoglobin regeneration as is ferric orthophosphate iron. It may be noted that McCance et al. ('43) observed in human subjects that the addition of disodium phosphate to bread decreased the absorption of iron. Caution must be exercised against confusing ferric orthophosphate itself with preparations in which the ferric orthophosphate has been solubilized with sodium citrate, e.g., Soluble Ferric Phosphate, N. F. VIII (National Formulary, '46).

The magnitude of the enrichment program justifies a thorough appraisal of the assimilability of the iron sources in use or proposed for use. Although the results of rat experiments are not applicable to man with certainty, these investigations with enriched bread strongly suggest the advisability of seeking better sources of iron than ferric orthophosphate for bread enrichment. The clinical findings of Moore and coworkers ('39) suggest that in man also ferric orthophosphate is poorly utilized as compared with ferrous sulfate. Certainly further studies upon the efficacy of ferric orthophosphate in both animals and man should be conducted if its use is to be continued. From a nutritional standpoint it would appear safer to use an iron source already known to be highly efficacious in man, such as ferrous sulfate, reduced iron, or other preparations of comparable availability. In order that the consumer may secure the full benefit of the enrichment program, it is desirable that highly assimilable forms of iron be used in bread and flour enrichment.

SUMMARY

The biological availabilities of the iron in ferrous sulfate and ferric orthophosphate have been compared on the basis of hemoglobin regeneration in rats made anemic from iron deficiency. A secondary comparison was made with ferric chloride. The iron compounds were fed in the form of enriched breads, and multiple levels of iron enrichment were used to permit comparison of dosage response curves.

Under the conditions of these experiments, ferrous sulfate iron was 4 to 5 times as available as ferric orthophosphate iron when both compounds were tested at 4 widely spaced levels.

When compared at 2 levels, ferric chloride iron was equal in biological availability to the highly effective ferrous sulfate iron, or 4 to 5 times as available as ferric orthophosphate iron.

Attention is called again to the desirability of using highly assimilable forms of iron in flour and bread enrichment, so that the consumer may secure the full benefit of the enrichment program.

ACKNOWLEDGMENT

The authors wish to express their appreciation to George Garnatz, of the Kroger Food Foundation, Cincinnati, Ohio, for the breads used in these experiments. The interest and criticism of Dr. M. L. Tainter and Dr. L. C. Miller are gratefully acknowledged. The hemoglobin determinations were made by Henry Rivenburg.

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MODIFICATION OF THE ACTION OF CAFFEINE ON THE SPONTANEOUS ACTIVITY OF THE WHITE RAT BY THE CONCOMITANT ADMINISTRATION OF VARIOUS FOOD MATERIALS[1, 2]

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SEVEN FIGURES

(Received for publication June 23, 1947)

Various investigators have found that caffeine above a certain threshold level has a stimulating action on the central nervous system (Hollingworth, '12; Cheney, '35; Horst and Jenkins, '35).

The present experiments are offered to show that this action of caffeine as manifested by an increase in the spontaneous activity of the albino rat, can be modified to an appreciable extent by certain food materials administered either simultaneously with the administration of caffeine or a short time afterward.

PROCEDURE

The method used for measuring spontaneous activity was that described by Schulte and his associates ('41). The apparatus consisted of a small wire cage measuring 6″ × 6″ × 6″ which was suspended from a spiral spring in such a manner

[1] Preliminary report, Fed. Proc., *6:* 119, 1947.

[2] The expense of this investigation was defrayed in large part by a grant-in-aid from the Sugar Research Foundation.

[3] Present address: Parke-Davis Co., Detroit, Michigan.

that all the movements of the animals, even the slightest ones, were summated by a work adder attached to the spring. A small pin inserted in the rim of the work adder dipped into a mercury cup upon each complete revolution thereby completing an electric circuit and activating a signal magnet writing on a slowly moving kymograph paper. Activity was measured and recorded in terms of the total number of revolutions of the work adder throughout the experiment. Twelve units which were set up in a small well-lighted room devoted exclusively at the time to the experiment were used simultaneously. All the experiments were run in the morning because at this time of day in a well-lighted room the activity of a white rat is at a minimum.

Seven sets of observations were made under different experimental conditions. Caffeine alkaloid in various amounts was administered in aqueous solution by stomach tube or intraperitoneally. In some experiments sugar dissolved in water was given by stomach tube simultaneously with or shortly after the administration of caffeine. In others peptone, vegetable oil, and the inert material agar were given instead of sugar. Further details of the procedure will be described under the following separate captions.

RESULTS

Experiment 1: Showing that caffeine causes an increase in spontaneous activity for several hours after administration whereas no permanent effect is produced by the daily consumption of a large amount of caffeine

Twenty-two animals were selected 1 week after weaning and given 75 mg caffeine/kilo daily in aqueous solution by stomach tube for 100 days. An equal number of litter mates were chosen for controls and by the same procedure given water daily instead of the caffeine solution. At the end of this period 24 hours were allowed to elapse without the administration of caffeine whereupon the animals were placed

in the activity cages for the first time. They were kept in the cages for 1 hour for the purpose of obtaining their basal activity, then removed and given caffeine or water by stomach tube and replaced in the cages. This procedure was repeated on successive days (except on Sundays) until 220 daily observations had been made on the test animals and the same number on the controls.

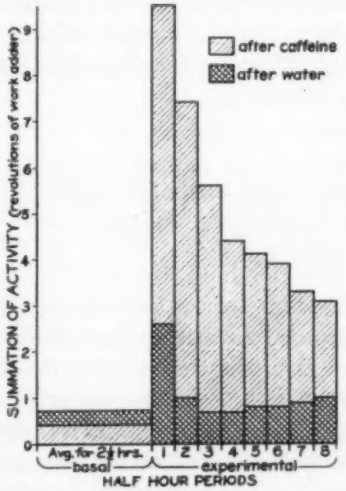

Figure 1

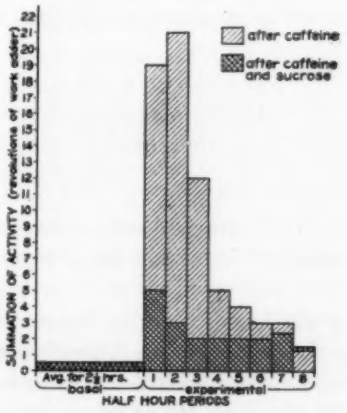

Figure 2

Fig. 1 The effect of 75 mg caffeine/kg on spontaneous activity. For the sake of continuity the activity of the animals under test and control conditions is represented by hatched and cross-hatched lines, respectively, although caffeine was not given to the test animals nor water to the controls until the end of the basal period.

Fig. 2 Comparison of spontaneous activity after caffeine in water and caffeine in a sucrose solution.

Seventy-five mg caffeine per kg body weight had a definite stimulating effect on spontaneous activity which was most marked during the first half hour. This effect was still manifest at the conclusion of the experiment 4 hours after the administration of caffeine. The results of the experiment are presented in figure 1. The activity of the test animals was

greater than that of the controls in 206 out of 220 individual experiments; in 5 experiments there was difference whereas in 9, the controls were slightly more active than the test animals.

Long continued daily administration of this amount of caffeine had no permanent effect as shown by the fact that the basal activity of the animals that had been given caffeine for 100 days was the same as that of the controls. In this connection it is of interest to note that in human subjects Cheney ('35) found no significant effect on reaction time 24 hours after the consumption of caffeine.

Experiment 2: Showing that sucrose administered simultaneously with caffeine reduces the increase in spontaneous activity caused by the stimulating action of caffeine

These experiments were undertaken with the purpose in mind of following up a lead obtained in other observations (not published) which suggested that the action of caffeine might be affected by the simultaneous ingestion of sugar.

In this and the following experiments each animal served as its own control. The animals were divided into 2 groups. On the first day of the experiment 1 group was studied under experimental and the other under control conditions. On alternate days thereafter this order was reversed.

Twelve adult rats were selected at random from our colony. Under control conditions the animals were given 20 mg caffeine/kg in aqueous solution by stomach tube and under test conditions the same amount of caffeine in a 50% sucrose solution (1.5 gm sucrose/100 gm body weight). It had been found previously in another set of experiments that 20 mg caffeine/kg had a definite stimulating action. These results are not presented separately as they are in agreement with those obtained with this amount of caffeine in the experiments shown in figure 2. Each point on the nomogram is plotted from an average of 60 experiments.

A comparison of the activity when caffeine alone was given with that which occurred after the simultaneous administration of caffeine and sucrose (fig. 2) shows that the sugar neutralized to a large extent the action of caffeine.

In other experiments, the results of which need not be given in detail, spontaneous activity after the administration of a sucrose solution alone was found to be practically the same as when plain water was given.

Experiment 3: Showing that the increase in activity caused by caffeine can be reduced by administering sucrose 30 minutes after giving caffeine

When it had been definitely shown in the preceding experiments that the ingestion of sucrose had a modifying effect on the action of caffeine, speculation arose as to the mechanism involved. To determine whether the effect of the simultaneous administration of sugar might be due to an interference with the absorption of caffeine the following experiments were undertaken.

The animals used in this experiment were the same as in experiment 2. The procedure and the amount of caffeine and sucrose administered were also the same with the exception that all the animals were given caffeine immediately after the basal period. Thirty minutes later those under test conditions were given the sucrose solution by stomach tube and those under control conditions were given the same amount of water. There were 48 observations under each set of conditions.

The area of cross-hatched lines in figure 3 for the first half hour after the basal period represents the activity of the animals on the days when they were given sucrose after this first half hour period; the hatched lines, the activity on the days when they were given water (control experiments). Within 30 minutes the caffeine had been absorbed and was exerting its maximum effect, as indicated by the activity of the animals at this time. Administration of sucrose was fol-

lowed in the next half hour period by a definitely greater decrease in activity than when water alone was given.

Experiment 4: Showing that caffeine injected intraperitoneally increases spontaneous activity which in turn is reduced by the oral administration of sucrose

This experiment is offered as confirmatory evidence that the effect of sucrose on the action of caffeine is not due to an interference with absorption. The observations were made on the same animals as in the preceding experiment.

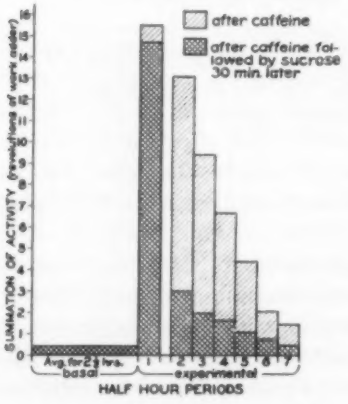
after caffeine
after caffeine followed by sucrose 30 min. later
SUMMATION OF ACTIVITY (revolutions of work adder)
Avg. for 2½ hrs. basal
experimental
HALF HOUR PERIODS

Figure 3

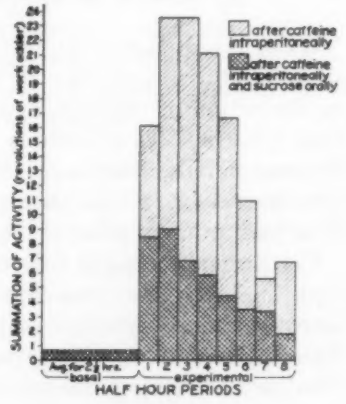
after caffeine intraperitoneally
after caffeine intraperitoneally and sucrose orally
SUMMATION OF ACTIVITY (revolutions of work adder)
Avg. for 2½ hrs. basal
experimental
HALF HOUR PERIODS

Figure 4

Fig. 3 Reduction in the increased activity produced by caffeine when sucrose was administered 30 minutes after caffeine.

Fig. 4 The action of the oral administration of sugar on the increase in spontaneous activity caused by the intraperitoneal injection of caffeine.

An aqueous solution containing 20 mg caffeine/kg was injected intraperitoneally into all the animals at the conclusion of the basal period. Immediately after the injection 1 group was given sucrose by stomach tube and the other water only. On alternate days this procedure was reversed.

The average values of the data obtained are plotted in figure 4. Each average was derived from 36 experiments.

During the first half hour after intraperitoneal injection of caffeine the activity was practically the same as in the preceding experiments in which caffeine was administered by stomach tube. In the second half hour there was a further increase in activity which continued at this high level during the following half hour period. From this time on there was a gradual return toward the basal level.

The oral administration of sugar neutralized to a large extent the stimulating action of caffeine that had been injected intraperitoneally.

Experiment 5: Showing that the increase in spontaneous activity induced by caffeine is reduced by the simultaneous administration of peptone

Further speculation on the mechanism whereby sucrose modifies the action of caffeine suggested the advisability of determining whether this effect was specific with respect to sucrose or common to other food materials of an entirely different nature. This experiment and experiment 6 were designed for this purpose.

Twelve animals that had not been used in the previous experiments were selected from the colony. The procedure was the same as in experiment 2 except that an aqueous solution containing 10 gm peptone/kg body weight was given instead of the sucrose solution.

The results presented in figure 5 show that peptone had an effect similar to that of sucrose in reducing the increase in activity caused by caffeine.

Experiment 6: Showing that administration of vegetable oil simultaneously with caffeine reduces the increased activity resulting from caffeine

Results similar to those in experiment 5 were obtained in the present experiment in which 20 mg caffeine dissolved in 3.75 ml vegetable oil was administered by stomach tube to 12 animals. The comparative effects of caffeine in water and

caffeine in oil are presented in figure 6. Each value from which the nomogram was constructed is an average of 60 experiments.

From this experiment and the preceding one it is apparent that the modifying effect on the action of caffeine which was observed originally in our studies with sucrose is not specific for sucrose but is shared by the other major foodstuffs, namely, protein and fat.

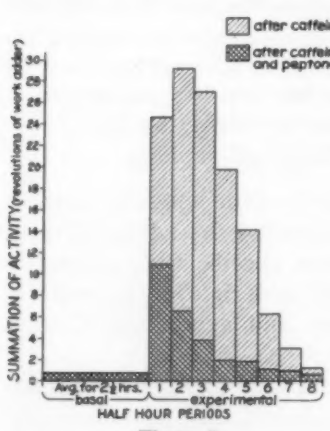

Figure 5

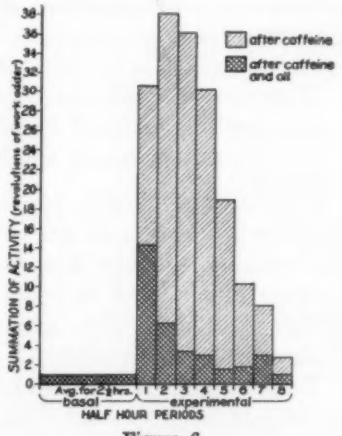

Figure 6

Fig. 5 Reduction in the stimulating action of caffeine by the simultaneous administration of peptone.

Fig. 6 The effect of the simultaneous administration of vegetable oil on the stimulating action of caffeine.

Experiment 7: Showing that the increase in spontaneous activity caused by caffeine is not affected by the simultaneous administration of inert material

There remained to be considered the possibility that the mere presence of materials other than caffeine in the gastrointestinal tract, regardless of their nature, might have an effect similar to that produced by the foodstuffs. This experiment, the results of which are presented in figure 7, shows that this is not the case. There was no significant difference in

the activity of the animals when given 20 mg caffeine/kg in aqueous solution by stomach tube and when given the same amount of caffeine dissolved in 10% agar. Each value plotted is an average of 30 observations.

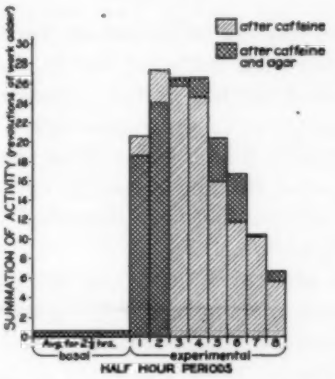

Fig. 7 Spontaneous activity following the administration of caffeine in water and caffeine in agar.

DISCUSSION

The original suggestion for the pursuit of this investigation arose in the course of another study still in progress which seemed to indicate that certain physiological actions of caffeine might be modified by the simultaneous ingestion of sucrose. The present study is concerned with a different underlying physiological mechanism.

While these experiments were under way it came to our attention that it had been reported by Chaucard ('45) and in another paper by the same author and his associates (Chaucard, Mazoue and Lecoq, '45) that the chronaxie of nerve fibers is affected by caffeine and that this effect is specifically neutralized by the oral administration of the sugars: lactose, sucrose, galactose and glucose.

When it had been proved to our satisfaction that the action of caffeine on the central nervous system can be modified by the administration of sucrose, our interest was aroused in

the mechanisms involved. The only contribution we can offer at the present time with regard to the question is of a negative nature as stated in the summary.

SUMMARY

1. The modification of the action of caffeine by sucrose is not specific to this food material but is shared by foods of an entirely different nature, namely, protein and fat.

2. It is not due to an interference with absorption for, as shown in experiments 3 and 4, the effect was observed when caffeine was injected intraperitoneally and also when sugar was given after caffeine had been absorbed and had produced its characteristic action.

3. It cannot be accounted for by the mere presence of other material regardless of its nature in the gastro-intestinal tract, for agar administered simultaneously with caffeine had no neutralizing action.

In respect to the lethal dose of caffeine our results differ from those of Schulte and his associates ('39). A dose of 40 mg caffeine/kg proved fatal in 1 of their 10 animals. Since 10 mg/kg sufficed to increase spontaneous activity it was concluded that the margin of safety between the barely effective and the convulsive or fatal dose was 4 times. In the experiments reported in this paper and in numerous other experiments we have administered 75 mg caffeine/kg over 9000 times to 145 different rats without a single fatality.

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SELF SELECTION OF DIET

V. APPETITE FOR CARBOHYDRATES [1]

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TWO FIGURES

(Received for publication June 16, 1947)

In a previous experiment, it was found that most young growing rats had little appetite for sucrose, when their choice of food was casein, fat, salt mixture, and sugar (Scott, '46). The present report gives the results of experiments in which other carbohydrates (starch, dextrin and lactose) were offered as choices instead of or in addition to sucrose.

EXPERIMENTAL

In each of the first 3 experiments, 10 male and 10 female weanling rats were placed in individual cages and allowed to eat a mixed diet [2] offered in all of 4 cups. The experiments differed in that the sources of carbohydrate in the diets were different. Raw corn starch was the carbohydrate in the first experiment, lactose in the second, and dextrin in the third. Vitamins were given separately as pills.[3] During a 3-week

[1] Contribution no. 640 of the Department of Chemistry, University of Pittsburgh. Aided by grants of the Nutrition Foundation, Inc., and the Buhl Foundation.

[2] The mixed diet consisted of 62% carbohydrate, 10% hydrogenated fat (Primex), 4% salt mixture (Jones and Foster, '42), and 24% casein (Labco "vitamin-free").

[3] One pill was given each rat daily. It contained: 60 μg thiamine hydrochloride, 120 μg riboflavin, 90 μg pyridoxine hydrochloride, 150 μg calcium pantothenate, 10 mg choline chloride, 1 mg α-tocopherol, and 55 I.U. vitamin A, and 11 I.U. vitamin D as 0.001 ml Natola; all in a dextrin-powdered sugar base.

control period, the amount eaten from each cup was recorded daily and the cups were then interchanged in a predetermined random manner. During a 3-week experimental period, the choices given the animals were fat, protein, salt mixture, and the same carbohydrate as in the control period, each in a separate cup. The amount eaten was recorded and the cups interchanged daily as before.

In the fourth experiment, 20 male and 20 female weanling rats were divided into 4 groups so that each animal had 1 littermate of the same sex in each of the other 3 groups. The members of each group were fed 1 of the following mixed diets in all of 4 cups during the control period: (1) 62% sucrose diet, (2) 62% starch diet, (3) 62% lactose diet, (4) 62% dextrin diet. In the experimental period all animals were given their choice in 4 separate cups of these 4 mixed diets.

In the final experiment, each of 20 rats was placed in a cage with 6 cups, all of which contained a diet consisting of 24% casein, 10% fat, 4% salt mixture, and 15.5% each of sucrose, starch, lactose and dextrin. During the experimental period 7 choices were allowed the animals. These consisted of casein, fat, salts, sucrose, starch, lactose and dextrin, each in a separate cup.

RESULTS

The growth and food consumption of the animals during the control period are shown in tables 1 and 2. All of the animals fed lactose diets developed severe diarrhea and grew very slowly. The other diets were approximately but not completely equivalent nutritionally as shown in table 2. The decreasing growth rates with these carbohydrates were: starch>dextrin>sucrose, with the difference between starch and sucrose definitely significant.

The results of the first 3 experiments and the 7-choice experiment are shown graphically in figure 1, while the carbohydrate choices in the latter experiment are shown in figure 2. The results of the experiment where mixed diets were offered are shown in table 3.

TABLE 1

Growth and food consumption in the control period.[1]

EXPT.	CARBOHYDRATE SOURCE	MALES		FEMALES	
		Wt. gain	Food eaten	Wt. gain	Food eaten
		gm	*gm*	*gm*	*gm*
1	Starch	104.5 ± 5.0	210.7 ± 10.4	82.7 ± 1.3	178.0 ± 6.2
2	Lactose	30.0 ± 3.3	116.8 ± 5.5	33.4 ± 4.2	110.1 ± 7.8
3	Dextrin	76.5 ± 4.1	190.4 ± 5.8	66.2 ± 3.7	176.1 ± 8.1
5	Mixture	70.7 ± 3.3	176.1 ± 4.6	62.3 ± 2.3	181.6 ± 4.9

[1] All data in terms of mean and standard error of the mean.

TABLE 2

Growth and food consumption during the control period.

(Experiment 4)

CARBOHYDRATE SOURCE	WT. GAIN	FOOD EATEN
	gm	*gm*
Sucrose	62.5 ± 3.6	133.0 ± 7.7
Starch	73.9 ± 3.6	161.8 ± 8.8
Lactose	31.2 ± 4.1	103.4 ± 6.0
Dextrin	68.7 ± 3.6	155.6 ± 5.2

TABLE 3

Preference for mixed diets.

CARBOHYDRATE SOURCE IN CONTROL PERIOD	WT. GAIN	TOTAL FOOD	APPETITE FOR DIET CONTAINING[1]			
			Sucrose	Starch	Lactose	Dextrin
Sucrose	78.7±7.4	248.9±17.2	—19.5±5.7	18.6± 7.2	—19.2±3.4	21.2±10.0
Starch	70.5±6.6	246.6±16.3	7.9±6.4	—12.6± 7.0	—23.0±2.1	27.0± 7.0
Lactose	81.9±3.6	225.6±10.6	—5.9±7.3	—1.0±10.2	—16.9±3.2	23.8± 9.7
Dextrin	67.9±5.8	225.3±19.7	16.1±7.0	2.3± 9.0	—25.8±5.1	7.2± 6.6
Average	74.8±3.0	244.1± 6.5	0.4±3.9	1.8± 4.4	—21.2±1.8	19.8± 4.2

[1] Change in percentage eaten (experimental minus control) from corresponding cups during control and experimental periods.

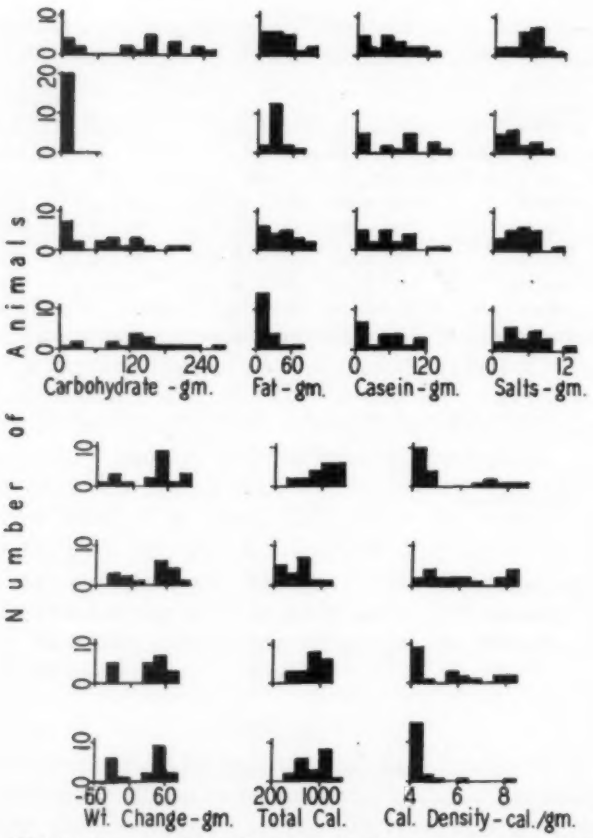

Fig. 1 Weight change and selections of animals when various carbohydrates were offered. The carbohydrate offered was in each case: Top histogram — starch; second histogram — lactose; third histogram — dextrin; lower histogram — sucrose, starch, lactose and dextrin offered simultaneously.

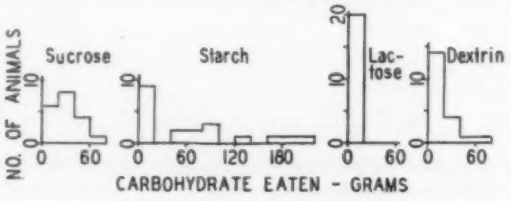

Fig. 2 Selection of carbohydrates when offered simultaneously.

DISCUSSION

In all of the present experiments lactose was thoroughly disliked. There was no obvious reason for this, but it is not inconceivable that the physiological response to this carbohydrate resulting in diarrhea may have caused unpleasant subjective symptoms in the animal. The other carbohydrates were accepted to a greater or less degree by some animals. The order of preference varied with the type of experiment,

TABLE 4

Effect of carbohydrate choice on protein choice.

CARBOHYDRATE CHOICE	NUMBER OF ANIMALS IN		PROBABILITY[2]
	Group A[1]	Group B	
Sucrose	34	53	1.000
Starch	5	15	0.214
Lactose	7	13	0.777
Dextrin	5	15	0.214
All four	7	13	0.777

[1] Group A ate little or no protein and lost weight; Group B selected protein and gained weight during the experimental period.

[2] Probability that an arrangement into groups as improbable as that shown could occur through random sampling. The theoretical probability of a rat eating protein was taken as that found in the sucrose experiment.

and the following orders of acceptability were observed: 1 carbohydrate as a choice (first 3 experiments), starch > dextrin > sucrose; 4 carbohydrates as choices (fifth experiment), starch > sucrose > dextrin; mixed diets as choices, dextrin > starch = sucrose.

The variation in intake of carbohydrate was accompanied by correlated inverse variation in fat. This is shown particularly well in the caloric density histograms, where it is seen that in those experiments where large amounts of carbohydrate were selected, the caloric density of the selections was low. Variation in carbohydrate intake had no observable effect on the number of animals which accepted protein as a choice, as shown in table 4. However, the complete avoidance

of lactose did result in some cases in high intakes of casein. This is apparent from the protein histogram, where it is seen that several animals ate large amounts of casein, in spite of the fact that, being smaller animals, their caloric intake was rather low.

For the most part, then, protein intake was independent of carbohydrate intake, but fat intake was intimately related to it. The 3 carbohydrates other than lactose were approximately equivalent nutritionally, and yet were accepted to different extents, and acceptance was dependent in part on how the choices were offered. It is improbable, therefore, that selection in these 3 cases was based on any nutritional qualities of the choices, and probable that the appetites shown for these substances were trivial in origin.

Furthermore, it appeared incorrect to speak of an "appetite for carbohydrate" since the appetites studied were essentially independent. The probability based on observation that an animal would not eat at least 20 gm of a given carbohydrate was as follows: sucrose 0.50; starch 0.20; lactose 1.00; dextrin 0.30. Assuming that the appetites were independent, the probability that an animal would not eat at least 20 gm of all 4 would be expected to be the product of the observed probabilities, or 0.03. Actually, only 1 out of 20 rats ate less than 20 gm of carbohydrate in the 7-choice experiment. The observed fraction was then 0.05, in close agreement with the expected probability.

The effect of previous diet on choice can scarcely be decided *a priori*, since one could as well expect animals to continue to eat the diet to which they are accustomed as to expect them to desire variety. It has been our qualitative impression from several experiments that rats relish a change of diet, although the effect only lasts for a day or two. This impression appeared to be substantiated by the data in table 3, where, in all cases but lactose, that group which liked a certain choice least was the group previously fed that choice.

SUMMARY

Of 4 carbohydrates tested in an experiment where rats were allowed to choose the components of a diet, lactose was avoided, but sucrose, starch, and dextrin were accepted to various degrees. The appetites for various carbohydrates were concluded to be largely independent of appetite for casein, inversely proportional to the intake of fat, independent (except that for lactose) of nutritional significance, dependent on the method of offering the choice, and independent of each other. Rats appeared to relish a change in the carbohydrate portion of their ration.

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PHYSIOLOGICAL AVAILABILITY OF THE VITAMINS

IX. INFLUENCE OF ASCORBIC ACID STABILIZERS IN FRUITS AND VEGETABLES [1]

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(Received for publication June 5, 1947)

In many homes fruit juices low in ascorbic acid are used interchangeably with those of higher ascorbic acid content. Some nutritionists have recommended that the former products be fortified with the vitamin to make them nutritionally more comparable with the higher potency juices. During and since the war, fortification of apple juice with ascorbic acid (35 mg per 100 ml) has been sponsored by the Canadian government (McFarlane and Davis, '41). In 1943–1945 the U. S. Army produced over 50 million pounds of canned apple juice in Australia, of which about 90% was fortified with synthetic ascorbic acid (Esselen, Powers and Fellers, '46). Enriched apple juice is now available on the American market. Procedures for the fortification of apple juice with ascorbic acid in commercial practice have recently been described by Hoffmann-LaRoche ('46), Merck ('46) and Pfizer ('46). In all these cases it has been the objective to obtain a fruit juice furnishing the minimum daily requirement, 30 mg

[1] The data in this report were presented before the American Institute of Nutrition, 1947, Chicago, Illinois. The expenses of the study were defrayed by a grant from the Duffy-Mott Company, Inc., New York, N. Y.

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of ascorbic acid (Federal Food, Drug and Cosmetic Act, '41) per serving of 100 or 120 ml. Furthermore, the vitamin has been reported to have a favorable effect on color retention of the fruit juice during storage (Esselen, Powers and Fellers, '46).

Interest in the vitamin C contribution of fortified apple juice was markedly stimulated by the recent report by Crampton and Burton ('46) that the vitamin potency is 35% greater than ascorbic acid content. These authors employed a new biological assay procedure, in which the maximum development in height of the odontoblast cells of the incisor teeth of young guinea pigs was the criterion of potency. Either of 2 factors could be responsible for the greater than theoretical values obtained; (a) an unknown factor in apple juice may act synergistically with ascorbic acid, permitting optimal absorption and utilization of the vitamin, or (b) the biological procedure is not specific for ascorbic acid, and some other compound naturally present in the juice exhibits a similar effect.

Studies suggesting that a mixture of flavone glycosides (citrin, or vitamin P) is required for the cure of certain hemorrhagic manifestations of scurvy have recently been summarized (Nutrition Reviews, '43, '44). Much of the evidence is based upon observations that natural foods rich in vitamin C are far more effective in the treatment of hemorrhagic conditions than equivalent amounts of synthetic ascorbic acid. Citrin, together with ascorbic acid, appears to be required for the maintenance of the integrity of the capillary wall.

On the other hand, it has been postulated (Elmby and Warburg, '37) that natural foods may contain an unknown factor, a covitamin, required for more effective utilization of ascorbic acid. The recent report by Crampton and Burton ('46) supports this hypothesis since the authors consider their newly developed bioassay procedure to be specific for ascorbic acid. They found that fruit juices (orange, apple and tomato) exhibit about 35 to 45% higher vitamin C potency than is

indicated by chemical assay of these materials, assays which included determination of dehydro- as well as reduced ascorbic acid. The reference material in the bioassays was the synthetic vitamin. That natural foods contain factors which inhibit oxidative loss of ascorbic acid has been reported by Somogyi ('45) and Reder ('46). The latter suggests that compounds containing the sulfur groups, -SS- and -SH-, in vegetable extracts induce greater retention of ascorbic acid when comparisons are made with the results of control tests of pure solutions of ascorbic acid (buffered or unbuffered) at the same pH value.

Studies of factors influencing the degree of availability of ascorbic acid from different sources have appeared in the recent literature. Tests with humans have shown that ascorbic acid oxidase, capable of rapid *in vitro* destruction of large quantities of vitamin C, is inactive in the digestive tract (Hochberg, Melnick and Oser, '45a). Apparently the enzyme is destroyed *in vivo* or its activity inhibited. Likewise the destructive effect of copper on ascorbic acid is not manifest in the digestive tract (Hochberg, Melnick and Oser, '45b). Thus, the importance of these 2 factors which could conceivably have been responsible for low values for available ascorbic acid can now be discounted. On the other hand, the possibility that ascorbic acid derived from fruits and vegetables exhibits a greater potency than the vitamin in pure solution still requires investigation. As the result of earlier studies it has been concluded that the ascorbic acid in orange juice (Hawley, Stephens and Anderson, '36), in raspberries (Todhunter and Fatzer, '40), in potatoes (Clayton and Folsom, '40), in cabbage and tomato juice (Clayton and Borden, '43), and in papayas and guavas (Hartzler, '45) is available to the human organism to the same degree as the synthetic vitamin in pure solution. However, in these investigations the basal dietaries were not free from other fruits and vegetables. The objective was to reduce the irrelevant ascorbic acid content to a minimum; despite this the dietaries still contributed from 5 to 20 mg of the vitamin per day. In view of the obser-

vation that certain vegetable extracts, even those low in ascorbic acid content, may contain vitamin C stabilizers (Somogyi, '45; Reder, '46) and since these may be responsible for enhancement of biological potency, it would seem quite likely that in the earlier studies the synthetic ascorbic acid taken with the basal dietary may also have been protected by the stabilizing factors present in the vegetables and fruits consumed. It is pertinent to point out in this connection that the protective factor in vegetables is not destroyed by boiling the extracts (Reder, '46).

The objectives of the study now being reported were to answer these questions: (1) is apple juice a favorable vehicle for ascorbic acid as a means of fortifying the human dietary, and (2) what influence do the stabilizing factors in fruits and vegetables have on ascorbic acid availability?

EXPERIMENTAL PART

In vitro study

That fruit juices contain ascorbic acid stabilizing factors has been demonstrated by the *in vitro* tests presented in table 1.

The analyses for ascorbic acid content were conducted according to the photometric procedure (Hochberg, Melnick and Oser, '43). Acidulated, but not buffered, deionized water was employed in the first test system. The second consisted of pasteurized apple juice at its natural pH, and the third of freshly expressed orange juice also at its natural pH. The ascorbic acid was added to the acidulated water and to the apple juice in amounts comparable to that found in the orange juice. The solutions were neither deaërated prior to fortification nor kept under nitrogen during solution of the vitamin. The samples were stored in cylindrical glass-stoppered jars under air with a head space of about one-tenth of the total capacity. At the end of 10 and 21 days' storage of the solutions at 45°C., 1 jar in each series was removed for analysis.[4] The

[4] No changes in pH occurred during storage of the samples.

values reported in table 1 are for total ascorbic acid, since very little dehydroascorbic acid was found. It will be noted from the results of these accelerated holding tests that the vitamin was appreciably more stable in orange juice than in apple juice, while in the latter, retention of ascorbic acid was far superior to that in pure aqueous solution.

TABLE 1

Stability of ascorbic acid in solution.

TEST SYSTEM [1]	TOTAL ASCORBIC ACID CONTENT			RETENTION OF ASCORBIC ACID	
	Freshly prepared [2]	Stored 10 days at 45°C.	Stored 21 days at 45°C.	Stored 10 days at 45°C.	Stored 21 days at 45°C.
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	%	%
Aqueous [3] solution of ascorbic acid at pH 3.35 (unbuffered)	56.0	7.3	0.3	13	0.5
Apple juice fortified with ascorbic acid, pH 3.30	51.4	34.3	4.6	67	9
Orange juice, freshly prepared, pH 3.40	56.1	48.3	13.4	86	24

[1] Ascorbic acid was added to the acidulated water and to the apple juice in amounts comparable to that naturally present in the orange juice. The solutions were neither deaërated prior to fortification nor under nitrogen during solution of the vitamin and subsequent storage. In each case (including the orange juice) the air head-space above the solutions in the cylindrical glass-stoppered jars was about one-tenth of the total volume.

[2] The solutions were held for a period of 2 hours at room temperature prior to the first series of assays.

[3] Deionized water.

In vivo studies

In line with the first objective of this investigation, an assay was conducted with human subjects to determine the availability of ascorbic acid in fortified apple juice in comparison with the availability of the vitamin when supplied by raw fruits and vegetables. Ascorbic acid oxidase in the latter products has already been shown to be inactive in the digestive tract (Hochberg, Melnick and Oser, '45a).

The assay method is based upon the observation that under standardized conditions the urinary excretion of ascorbic acid parallels the quantity consumed (Melnick, Hochberg and Oser, '45). The test subjects of the preceding study were employed in the current investigation. They were healthy adult males, subsisting regularly on adequate, well-balanced dietaries. They excreted normal quantities of ascorbic acid before and after the administration of an oral test dose.

In the first availability study, comparison was made between the availability of ascorbic acid in raw fruits and vegetables (Control Period) and that derived solely from the fortified apple juice (Test Period). The ration consumed by the subjects during the Control Period was that routinely employed (Melnick, Hochberg and Oser, '45), modified only in that a greater amount of raw vegetables was included.[5] The values for the proximate constituents and vitamin content of this diet were the same as those described in the earlier paper (Melnick, Hochberg and Oser, '45).

The ascorbic acid-free basal ration, prior to supplementation with fortified apple juice, is described in table 2. In order to avoid the criticism directed at preceding studies, the diet was formulated free of all fruits and vegetables. The proximate and vitamin composition[6] of the unsupplemented diet was practically the same as that of the adequate basal ration except in ascorbic acid content. However, the addition of enriched apple juice, 460 ml in one-third portions at the end of each meal, contributed 115 mg of ascorbic acid, the same as that derived from the adequate diet containing raw fruits and vegetables.

During each period the subjects subsisted on the dietary for 2 weeks; the first 10 days constituted the adjustment period during which no urinary collections were made. Twenty-four-hour urine samples were collected during each of the next 4

[5] The ration included bread, butter, steak, milk, eggs, sugar, oranges, bananas, apples, lettuce, tomatoes, cucumbers, and a salad of raw cabbage, green beans, pepper and carrots.

[6] The methods employed for analyzing the rations were the same as those used in the preceding study (Melnick, Hochberg and Oser, '45).

days. On the final day, following the noon meal, a 200-mg test dose of extra ascorbic acid in aqueous solution was ingested. The precautions taken in collecting the urine samples to prevent loss of ascorbic acid have already been described (Melnick, Oser and Hochberg, '45). The photometric pro-

TABLE 2

Ascorbic acid-free diet.

	Breakfast		
2 slices of toast (wheat)	50 gm	1 cup of coffee	180 gm
2 butter squares	14 gm	(10 gm sugar)	
		(30 gm milk)	
	Dinner		
1 steak (lean)	200 gm	1 bottle of beer	360 gm
3 slices of bread (rye)	97 gm	1 cup of coffee	180 gm
3 butter squares	21 gm	1 serving of cake	55 gm
	Supper		
Noodle soup mix [1]	31 gm	3 butter squares	21 gm
2 fried eggs	81 gm	½ bottle of beer	180 gm
Cheddar cheese	70 gm	1 cup of coffee	180 gm
2 slices of bread (rye)	65 gm	1 serving of cake	55 gm
	Vitamin supplements [2]		
Thiamine	0.5 mg	Riboflavin	1.0 mg

Analyses conducted on aliquots of the composite diet

Proximate analysis	*Values found* [3]	*Vitamin content*	*Values found* [3]
Total weight	1840 gm	Ascorbic acid	0 mg
Total solids	521 gm	Thiamine	1.48 mg
Moisture	1319 gm	Thiamine: Calorie ratio	0.6
Protein	100 gm	Thiamine: Non-fat calorie ratio	1.0
Fat (ether extract)	130 gm	Riboflavin	2.40 mg
Ash	21 gm	Nicotinic acid [4]	19.8 mg
Crude fiber	3 gm		
Carbohydrate (by difference)	267 gm		
Calorie value	2640 cal.		
Non-fat calories	1470 cal.		

[1] A mixture of glucose, monosodium glutamate, salt, spices, hydrogenated fat, chicken fat, and noodles.

[2] One-third aliquots taken at the end of each meal.

[3] Expressed in terms of total food consumed in the 3 meals.

[4] Predominantly as the amide.

cedure for estimating the vitamin content (Hochberg, Melnick and Oser, '43) included measurement of both dehydro- and reduced ascorbic acid. In this report only the total ascorbic acid values are presented, since in all cases approximately 90% of the vitamin in the urine was present as reduced ascorbic acid.

TABLE 3

Urinary excretion of dietary and extra ascorbic acid by subjects ingesting raw fruits and vegetables as compared with fortified apple juice[1] as the sole source of dietary vitamin C.

SUBJECT	DIET CONTAINING RAW FRUITS AND VEGETABLES				DIET CONTAINING FORTIFIED APPLE JUICE			
	Basal excretions			After 200-mg test dose of ascorbic acid	Basal excretions			After 200-mg test dose of ascorbic acid
	1st 24 hours	2nd 24 hours	3rd 24 hours		1st 24 hours	2nd 24 hours	3rd 24 hours	
	mg	*mg*	*mg*	*mg/1st 24 hours*	*mg*	*mg*	*mg*	*mg/1st 24 hours*
J. C.	36	45	57	141	48	41	42	139
E. M.	74	61	59	172	53	44	50	141
D. M.	40	44	43	163	41	46	37	96
M. H.	67	59	57	199	36	28	31	141
H. H.	22	23	27	103	36	52	39	121
Average	48	46	48	156	43	42	40	128

[1] Fortified from its original content of 0.7 mg to 30 mg of ascorbic acid per 120 ml. The fortified apple juice (460 ml per day) was taken in one-third portions at the end of each meal. The total intake of ascorbic acid was 115 mg per day, the same as that derived from the diet containing the raw fruits and vegetables.

In table 3 are listed the urinary excretion values obtained during the Control (raw fruit and vegetable) and Test (fortified apple juice) Periods. The differences in both the average basal and the test dose excretion values for the 2 periods were found not to be statistically significant ($t = 0.8$ and 1.5, respectively).[7] It may therefore be concluded that the ascorbic acid in the enriched apple juice is available to the same extent as the vitamin contributed by the raw fruits and vegetables in a natural, adequate diet.[8] These data, together with the

[7] A t-value of 2.3 would indicate that the difference between the averages of 2 sets of 5 observations is significant (95% probability).

[8] Considering that the average urinary excretion value for the 5 standardized subjects is reproducible to within $\pm 10\%$ (Melnick, Hochberg and Oser, '45), it is apparent that the figures for comparative availability of the ascorbic acid in the fortified apple juice fall within the limits of precision of the biological assay.

results of the *in vitro* accelerated holding tests (see table 1), support the view of some nutritionists that apple juice is a favorable vehicle for supplying ascorbic acid to the human dietary.

The results of the assay presented do not answer the question whether or not the stabilizing factors occurring naturally in fruits and vegetables augment ascorbic acid availability. In both the control and test periods sufficient quantities of the unknown stabilizing agent may have been supplied to permit optimal absorption and utilization of the vitamin. That apple juice also contains factors which favor ascorbic acid stability has been demonstrated in tests previously described. To investigate the effect of the stabilizers, it is necessary that the ascorbic acid be taken during 1 of the periods in pure aqueous solution along with a basal ration free of all fruits and vegetables. Such a study was, therefore, also conducted.

In order to accentuate possible differences in urinary excretion values, the basal ration was supplemented with only 50 mg of ascorbic acid. This quantity in a natural diet is generally considered to be well above the minimum daily requirement (Federal Food, Drug and Cosmetic Act, '41). It is conceivable that when the ascorbic acid is taken in pure aqueous solution as a supplement to the ascorbic acid-free diet there might be negligible excretion of the vitamin in the urine, whereas administration of the vitamin in the apple juice containing stabilizing factors might be followed by appreciable excretion of the vitamin. If, in the absence of the stabilizing factors, excessive destruction of ascorbic acid occurs *in vivo,* depletion of tissue stores might result. This would be particularly evident from the excretion values obtained following dosage with the 200-mg test dose of extra ascorbic acid.

The time schedule of the second study was similar to that in the first. The same period was allowed for adjustment to the intake of 50 mg of ascorbic acid. Four consecutive urine samples were then collected. The first 3 were analyzed for estimation of the basal excretion value; the fourth for the de-

termination of the tissue stores of this vitamin as reflected by urinary excretion following postprandial ingestion of a 200-mg test dose of extra ascorbic acid. During the interval between the 2 urinary excretion studies, the subjects subsisted on a customary adequate diet furnishing approximately 115 mg of ascorbic acid per day. The urinary excretion values demonstrated that such an interval was ample to allow the subjects, on completion of the first portion of this study, to return to the state of nutrition they were in prior to the second phase.

TABLE 4

Urinary excretion of dietary and extra ascorbic acid by subjects ingesting a vitamin C-free diet supplemented with fortified apple juice [1] or with a pure aqueous solution of ascorbic acid.

SUBJECT	DIET SUPPLEMENTED WITH FORTIFIED APPLE JUICE [2]				DIET SUPPLEMENTED WITH AQUEOUS ASCORBIC ACID SOLUTION [2]			
	Basal excretions			After 200-mg test dose of ascorbic acid	Basal excretions			After 200-mg test dose of ascorbic acid
	1st 24 hours	2nd 24 hours	3rd 24 hours		1st 24 hours	2nd 24 hours	3rd 24 hours	
	mg	*mg*	*mg*	*mg/1st 24 hours*	*mg*	*mg*	*mg*	*mg/1st 24 hours*
J. C.	15	13	8	32	14	17	13	52
E. M.	11	13	15	50	13	7	7	18
D. M.	12	12	12	24	18	16	12	46
M. H.	9	9	4	16	11	14	10	29
F. O.	11	15	8	47	16	16	15	58
Average	12	12	9	34	14	14	11	41

[1] Fortified from its original content of 0.7 mg to 30 mg of ascorbic acid per 120 ml.

[2] Both supplements furnished 50 mg of ascorbic acid per day.

In table 4 are listed the urinary excretion values obtained in the second availability study. Here also there was little difference in either the basal excretion figures or in the total values following dosage with extra ascorbic acid. No greater destruction occurs *in vivo* when the vitamin is taken in pure aqueous solution free of the stabilizing factors in fruits and vegetables. Indeed, calculations of the availability of ascorbic acid in the fortified apple juice gave values appreciably less than 100% when compared with the data obtained after dosage

with the aqueous vitamin solutions. The differences in both the average basal and the test dose excretion values for the 2 periods are, however, not statistically significant ($t = 1.2$ and 0.5, respectively).[9] It may therefore be concluded that the stabilizing factors in apple juice responsible for greater stability *in vitro* do not increase the availability of ascorbic acid to the organism. It would also follow that the enhanced biological potency of ascorbic acid in apple juice claimed by Crampton and Burton ('46) is not due to a factor which permits greater absorption and more effective utilization of the vitamin, but possibly to the use by these investigators of an assay procedure which is not specific for ascorbic acid alone but also estimates the activity of a covitamin (possibly citrin) required for the development of the odontoblast cells of the incisor teeth of guinea pigs.

Since in the first assay comparing the availability of ascorbic acid in fortified apple juice with that in a dietary containing raw fruits and vegetables, no significant differences in urinary excretion responses were observed, one may generalize by saying that the stabilizing factors in fruits and vegetables which protect ascorbic acid *in vitro* are ineffective, in the digestive system, in increasing ascorbic acid availability. Either the period for this effect to become apparent *in vivo* is too short or other protective factors may be present in the basal ration or in the digestive juices which mask the activity of the ascorbic acid stabilizing factors supplied by fruits and vegetables.

SUMMARY

Ascorbic acid is far more stable in fruit juices than in aqueous solutions. However, the stabilizing factors are ineffective in the digestive system in increasing ascorbic acid availability. On the basis of both *in vitro* and *in vivo* studies, it is concluded that apple juice is a favorable vehicle for ascorbic acid in the human dietary.

[9] It is to be expected that the reproducibility of the excretion responses would be poorer when the urinary ascorbic acid values are small.

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VITAMIN A INTAKE IN CATTLE IN RELATION TO HEPATIC STORES AND BLOOD LEVELS[1]

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ONE FIGURE

(Received for publication June 4, 1947)

The relation of vitamin A intake in animals to hepatic stores and blood levels has been reported in a number of papers. Lewis et al. ('42) determined hepatic stores and blood levels of vitamin A in rats after 6 weeks on a diet containing from 0 to 1000 I.U. of vitamin A daily. Vitamin A blood levels were found to reach a maximum with a daily intake of 100 I.U. Hepatic stores of vitamin A increased with increased intake throughout the range of vitamin A fed. Caldwell et al. ('45) studied hepatic stores of rats receiving 3, 6, and 12 I.U. of vitamin A per gm of dry ration during an experimental period of 300 days. The animals receiving 6 and 12 I.U. showed increasing hepatic stores of vitamin A throughout the experimental period. Braun ('45) studied hepatic stores and blood levels of carotenoids and vitamin A as affected by seasonal change and rations. Seasonal change in blood vitamin A levels was found to depend upon the carotene and vitamin A intake. Significant differences were found in hepatic stores of vitamin A in 3 of 4 dietary groups studied. Baumann et al. ('34) showed that 95% of the vitamin A stores in the rat are to be found in the liver, providing the body stores of vitamin A are adequate. Guilbert and Hart ('35),

[1] Colorado Agricultural Experiment Station, Scientific Series Article no. 249.

working with cattle, found 93% of the body stores of vitamin A in the liver. The principal stores of carotene were found in the body fat. Frey and Jensen ('46) showed that a rapid decrease in hepatic stores of vitamin A and carotene occurred in cattle on a fattening ration consistent with good feeding practice. Riggs ('40) has shown that night blindness will develop in cattle in from 46 to 266 days on a ration practically devoid of carotene. The time required to produce night blindness was found to depend upon age, the nature of the ration, and the ration fed previously.

In the present paper data are presented on hepatic stores and blood serum levels of vitamin A and carotene in cattle on a carotene-free basal ration containing varying amounts of vitamin A in the form of high potency fish oil. The paper represents in part an investigation of the cause of liver abscess formation in cattle. The experiment was based on data derived from 2 previous experiments (Frey and Jensen, '46; Jensen et al., '47).

EXPERIMENTAL DESIGN AND RESULTS

One hundred and fourteen Hereford steers of about 8 months of age were taken from native grass pasture and divided into 6 lots of 19 animals each. Lot 1 was slaughtered at the beginning of the experiment to determine the initial stores of vitamin A and carotene in the blood and liver. The remaining 5 lots were placed on experimental rations December 7, 1945. Table 1 gives the basal ration and vitamin A supplement for each lot. The rations were hand fed in amounts demanded by the animals for maximum consumption.

The vitamin A supplement was prepared by mixing a high potency oil, guaranteed to contain not less than 200,000 I.U. of vitamin A per gm, with a low potency oil to give a mixture containing approximately 25,000 I.U. per gm. The vitamin A content of the mixture was determined colorimetrically by means of the Carr-Price reaction (Carr and Price, '26) using an Aminco type-F photoelectric colorimeter. Crystalline vitamin A alcohol was used as the reference standard. One μg

of vitamin A alcohol was considered equivalent to 4.3 I.U. of vitamin A. The vitamin A-containing oil was mixed well with the rolled barley, this in turn being mixed with the other ration constituents in the feed trough. The animals were weighed at 28-day intervals and the vitamin A supplement adjusted to give the intake per pound weight of animal as given in table 1.

TABLE 1

Vitamin A supplements, average daily rations, weight gains, and grade of carcass.

LOT. NO.	2	3	4	5	6
I.U. vitamin A/lb. body wt. daily	0	25	100	200	500
Average daily rations (lb.)					
Rolled barley	4.56	5.58	5.55	5.58	5.58
Dried beet pulp	4.47	5.47	5.44	5.47	5.47
Wet beet pulp	4.88	5.42	5.44	5.41	5.39
Ground oat straw	2.43	2.62	2.62	2.66	2.65
Cottonseed meal	0.94	0.99	0.99	0.99	0.99
Mineral	0.03	0.03	0.05	0.05	0.04
Salt	0.04	0.05	0.05	0.07	0.06
Average initial weight (lb.)	460	461	460	461	460
Average weight — 166 days (lb.)	760	846	834	846	849
Average weight — 277 days (lb.)	842	999	993	1000	1025
Average daily gain (lb.)	1.52	2.07	2.03	2.07	2.14
Government grade of carcass					
Choice	6	11	9	12	11
Good	9	8	10	7	8
Commercial	3	0	0	0	0
Condemned	1	0	0	0	0

Blood samples were taken from each lot of animals at 27 and 83 days. Nine animals from each lot were slaughtered after 166 days of the experiment and the remaining 10 at 280 days. Liver tissue was obtained from each animal at the time of slaughter. Blood samples were taken from the animals at the last weighing previous to slaughter, corresponding to 159 and 277 days, respectively. The sampling technique and methods of analysis for vitamin A and carotene in liver tissue were the same as described by Frey and Jensen ('46). Serum vitamin A and carotene were determined by the method of

Kimble ('39). The ration constituents contained no carotene or preformed vitamin A.

DISCUSSION OF RESULTS

In table 1 are given the average daily consumption of ration constituents and the weight gains in pounds for each lot during the experimental period of 277 days. The animals in lot 2 show an average daily gain of 1.52 pounds as compared to an average of 2.08 pounds for the other 4 lots. The carcasses in lot 2 showed less finish and graded lower than did those in the other lots. The 4 lots receiving vitamin A supplement graded about equally, indicating that an intake of 25 I.U. of vitamin A per pound body weight daily is sufficient to maintain finish.

Indications of vitamin A deficiency were observed in the animals in lot 2 during the fourth month of the feeding period. Typical eye involvement with impaired vision, as manifested by some change in the ability to discern objects in daylight, was observed in 8 of the 10 animals in lot 2 at 277 days.

The data in table 2 give the average values found for serum levels and hepatic stores of vitamin A and carotene for each lot while on the experimental rations. The serum levels of carotene decreased rapidly. At the 83-day period and thereafter no trace of carotene was found in the serum. This is in contrast to the hepatic stores where definite amounts of carotene were found at 280 days. At the end of 27 days the carotene content of the serum had dropped to approximately $\frac{1}{2}$ the initial value, there being no significant difference between lots. In lot 2 the serum values of vitamin A reached a maximum at 27 days. Subsequent values at 159 and 277 days showed a rapid decrease of vitamin A. Lot 3, which received 25 I.U. of vitamin A per pound body weight daily, showed increasing serum values up to 83 days. Between 83 and 159 days the serum levels of vitamin A started decreasing. Lot 4, which received 100 I.U., showed increasing serum levels of vitamin A for 159 days. Lot 5, which received 200 I.U., and lot 6, which received 500 I.U. showed similar patterns. Each

TABLE 2

Serum levels and hepatic stores of vitamin A and carotene expressed as micrograms of constituent per 100 ml of serum or per gm of liver.[1,2]

DAYS ON RATION	LOT 2			LOT 3			LOT 4			LOT 5			LOT 6		
	$\bar{x}$	σ	η	$\bar{x}$	σ	η	$\bar{x}$	σ	η	$\bar{x}$	σ	η	$\bar{x}$	σ	η
							Serum vitamin A								
0[3]	30.0	7.1	17												
27	34.4	8.2	13	34.7	5.7	11	39.0	6.8	14	42.0	6.8	17	38.0	9.5	14
83	34.4	12.1	9	43.5	15.5	10	39.4	7.0	9	42.6	11.1	10	44.6	11.1	11
159	13.7	2.1	9	40.2	4.0	9	52.7	4.6	9	50.4	7.8	9	53.7	8.1	9
277	5.2	2.3	10	29.2	6.0	10	48.6	5.1	10	55.1	8.5	10	57.2	8.8	10
							Serum carotene								
0[3]	44.5	12.5	17												
27	29.4	18.7	13	23.3	11.6	11	21.6	7.2	14	23.4	13.3	17	25.1	11.2	14
							Hepatic vitamin A								
0[3]	47.1	22.7	16												
166	0.4	0.3	9	2.6	1.5	9	18.4	4.8	9	52.6	25.3	9	127.0	32.2	9
280	0.2	0.1	10	1.4	0.8	10	17.4	7.1	10	40.0	10.4	10	130.0	50.9	10
							Hepatic carotene								
0[3]	3.6	2.3	16												
166	0.68	0.33	9	0.65	0.19	9	0.56	0.12	9	0.70	0.26	9	0.48	0.17	9
280	0.35	0.28	10	0.29	0.12	10	0.29	0.14	10	0.22	0.07	10	0.26	0.19	10

[1] $\bar{x}$ = Arithmetic mean of values found; σ = standard deviation; η = number of animals from which tissue samples were obtained.

[2] See table 1 for vitamin A supplement given each lot.

[3] Serum levels and hepatic stores at 0 days the same for all lots.

showed increasing levels of serum vitamin A throughout the experimental period of 277 days. An intake of 200 I.U. or more of vitamin A per pound body weight daily was sufficient to meet requirements in addition to increasing serum levels of vitamin A. Under these experimental conditions an intake of between 100 I.U. and 200 I.U. of vitamin A per pound body weight daily maintained a nearly maximum serum level.

The increasing pattern of serum vitamin A in lots 2, 3 and 4 up to 27, 83 and 159 days, respectively, is difficult to explain. The animals had been shipped from the Texas Panhandle and placed on native grass pasture about 3 weeks prior to being placed on experimental rations. It is probable that the animals were in the process of building up hepatic stores of carotene and serum vitamin A at the beginning of the experiment. One would expect a time factor to be involved in establishing blood levels of vitamin A from hepatic stores of carotene when the latter are increasing. Since these conditions most likely prevailed it is possible that the relatively large hepatic reserves of carotene were responsible for the increasing serum levels of vitamin A that were observed.

At the end of 166 days the average hepatic stores of carotene had dropped from 3.6 μg per gm of liver to 0.61 μg. At the end of 280 days the average carotene content was 0.28 μg per gm of liver. As previously shown by Frey and Jensen ('46), the rate of depletion of hepatic stores of vitamin A and carotene diminishes with decreasing hepatic stores of either of the 2 constituents. Hickman ('46) postulated the existence of a half-time recovery or depletion period for body stores of a vitamin. On the basis of such a theory the greatest loss of carotene stores would be predicted to occur during the initial period of the experiment. The small difference in the hepatic reserves of carotene for the different lots at 166 and 280 days indicates that vitamin A does not exert an appreciable sparing action on hepatic stores of carotene.

The vitamin A content of the livers of the animals in lot 2 was found to be 0.2 μg per gm of liver after 280 days on a carotene-free ration. The significance of such a low value is

questionable when one considers the influence of interfering substances in the Carr-Price reaction. The animals receiving 200 I.U. or less of vitamin A per pound body weight daily showed decreased hepatic stores of vitamin A at the end of the experimental period of 280 days. Those receiving 500 I.U. showed increased hepatic stores at the end of the experimental period. No significant increase in hepatic stores of vitamin A was found between 166 and 280 days.

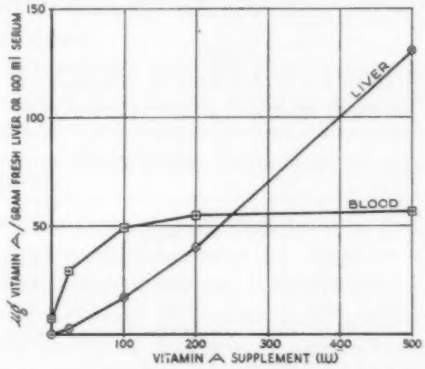

Fig. 1 Hepatic stores at 280 days, and blood serum levels at 277 days, of vitamin A compared with varying vitamin A supplements in I.U. per pound body weight daily.

In figure 1 hepatic stores of vitamin A at 280 days and serum levels of vitamin A at 277 days are compared with varying vitamin A supplements (tables 1 and 2). It will be observed that hepatic stores of vitamin A increase in practically a linear relationship with vitamin A intake. The serum levels of vitamin A show a rapid increase up to an intake of 100 I.U. of vitamin A per pound body weight daily. Above an intake of 200 I.U. the rate of increase in serum vitamin A is small. This suggests that an intake of 100 I.U. of vitamin A per pound body weight daily is sufficient to maintain nearly maximum serum levels in cattle under the conditions of the experiment. This is supported by reference to hepatic stores of vitamin A as given in table 2. Hepatic stores of vitamin A

began increasing above the initial values between an intake of 200 I.U. and 500 I.U. of vitamin A per pound body weight daily when the initial reserves were as given in table 2.

Figure 1 shows no simple relationship to exist between serum levels and hepatic stores of vitamin A. This suggests that the body mechanism controlling serum levels of vitamin A is different from that controlling hepatic stores.

Table 3 summarizes the results obtained in making a comparison of individual values between lots to determine the

TABLE 3

Orthogonal comparison of lots to determine the significance of blood and liver values of vitamin A as given in figure 1.[1]

LOTS COMPARED	σ	$\sigma\bar{x}$	M.S.D.	
			0.05	0.01
		Blood — 277 days		
2 and 3	4.17	1.87	3.90	5.34
3 and 4	2.00	0.90	1.87	2.55
4 and 5	8.84	3.95	8.27	11.30
5 and 6	41.00	18.40	38.50	52.60
		Liver — 280 days		
2 and 3	0.51	0.23	0.48	0.66
3 and 4	5.10	2.28	4.78	6.53
4 and 5	10.30	4.60	9.62	13.20
5 and 6	32.50	14.52	30.40	41.60

[1] σ = Standard deviation; $\sigma\bar{x}$ = standard error; M.S.D. = minimum significant difference between means at the 5 and 1% levels.

significance of the average values shown in figure 1. The values for the minimum significant differences between means show that for blood levels of vitamin A the first 2 orthogonal comparisons differ very significantly, while the last 2 do not differ significantly. This indicates that blood levels of vitamin A, under the conditions of the experiment, reach a maximum value at an intake of between 100 I.U. and 200 I.U. of vitamin A per pound body weight daily. For the liver values of vitamin A all 4 orthogonal comparisons show highly significant differences. This indicates that hepatic stores of vitamin A increase

with increasing intake of vitamin A throughout the range fed in the experiment.

SUMMARY AND CONCLUSIONS

Hereford steers of about 8 months of age were maintained on a carotene-free basal ration containing vitamin A supplements at levels of 0, 25, 100, 200 and 500 I.U. per pound body weight daily. Serum levels of vitamin A and carotene were determined at 0, 27, 83, 159 and 277 days, and hepatic stores at 0, 166 and 280 days.

The animals receiving only the basal ration showed a lower ration consumption and lower average daily gain than those receiving vitamin A supplement. The ration consumption and weight gains were about the same for all lots receiving vitamin A supplement.

Dietary vitamin A did not exert a sparing action on hepatic stores of carotene. Blood stores of carotene were depleted sooner than were hepatic stores. Hepatic stores of vitamin A increased in practically a linear relationship with intake throughout the range of vitamin A supplement fed. Serum levels increased rapidly up to an intake of 100 I.U. of vitamin A per pound body weight daily. An intake of 100 I.U. of vitamin A per pound body weight daily will maintain nearly maximum serum levels of vitamin A in cattle under the conditions of the experiment.

Serum levels and hepatic stores of vitamin A appear to be controlled by different body mechanisms.

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STUDIES OF THYROID TOXICITY

I. A NUTRITIONAL FACTOR WHICH ALLEVIATES THE TOXICITY OF INGESTED THYROID SUBSTANCE [1]

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(Received for publication June 20, 1947)

The importance of nutrition in experimental hyperthyroidism and its effect on the course of the syndrome have been emphasized by many investigators. The vitamin requirements of hyperthyroid animals appear to be increased. Abelin ('30) found that increasing the casein content of a thyrotoxic diet decreased the severity of the symptoms. The casein used was a crude preparation and may have carried other essential nutrients with it. There is a great deal of evidence which indicates an increased requirement for vitamin A (Abelin et al., '30; Abelin, '33; von Euler and Klussman, '32; Logaras and Drummond, '38; Sure and Buchanan, '37b) and in guinea pigs for ascorbic acid (Svirbely, '35; Sure and Theis, '39) during experimental hyperthyrodism.

Most attention has been focused on the vitamins of the B complex. These vitamins, where their function has been elucidated, have been found to be associated in the enzyme systems which yield energy to the organism. The thyroid gland through its hormone appears to exert a regulatory influence on these energy-yielding metabolic processes. At present, the specific function of the thyroid hormone is not clear.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

It is quite logical to assume that an increased metabolic rate which results from the administration of a thyrotoxic material may necessitate an increased dietary intake of members of the B complex. The work of many workers confirms such a hypothesis (Abelin, '30; Cowgill and Palmieri, '33; Himwich et al., '31, '32). The partial protective effect exerted by vitamin B_1 was first shown by Sure and Smith ('34), Sure and Buchanan ('37a) and confirmed by Drill and Sherwood ('38). Thyrotoxicity has been shown to be accompanied by a reduction of vitamin B_1 content of the tissues (Drill, '37) and also of their cocarboxylase activity (Peters and Rossiter, '39). Vitamin B_1 alone did not give as adequate protection as was obtained when yeast or a yeast concentrate was added to the ration (Drill, '37; Sure and Smith, '34; Sure and Buchanan, '37a).

As more members of the B complex became available in pure form, their effects in counteracting thyrotoxicity were studied. Drill and Overman ('42) found that pyridoxine and calcium pantothenate were required in larger quantities during experimentally induced thyrotoxicosis. Their ration contained a minimal quantity of yeast. Abelin ('45, '46), feeding a crude ration, observed a beneficial effect when massive doses of calcium pantothenate were administered to hyperthyroid rats. Ershoff and Hershberg ('45) were unable to alleviate the symptoms resulting from thyroid administration by greatly increasing the calcium pantothenate level of the ration. Katzenelbogen, Alexrod and Elvehjem ('41) observed that increasing the nicotinic acid content of the ration did not affect the gross symptoms of thyrotoxicity, but did prevent the accompanying decrease in the coenzyme I content of the tissues.

Ershoff and Hershberg ('45) found that rats fed a purified ration very high in the members of the vitamin B complex known to be required by the rat grew poorly when desiccated thyroid was added. The feeding of a yeast-containing ration resulted in much better growth and survival. This indicated that some factor present in yeast, other than the vitamins

added, exerted an antithyrotoxic effect. Their purified ration did not contain inositol, biotin, nicotinic acid, folic acid, and p-aminobenzoic acid.

The present investigation was undertaken to determine the nature of the growth stimulating, antithyrotoxic agent in yeast and other sources. It was designed to show whether this effect was due to one of the known members of the vitamin B complex or whether an as yet unknown factor was responsible.

PROCEDURE

Male, weanling albino rats of the Sprague-Dawley strain and weighing 35–45 gm were used in this study. It has been shown by earlier work (Drill, '38a, b) that male rats are more sensitive to administered thyroid substance than are females.

TABLE 1

Composition of the rations.

	RATION A	RATION B
	%	%
Casein (hot alcohol extracted)	22	22
Sucrose	68.5	56.5
Salts IV (Phillips and Hart, '35)	4.5	4.5
Corn oil	5	5
Dried brewer's yeast	..	12

To each kg of the above rations the following crystalline vitamins were added: thiamine hydrochloride 3 mg, riboflavin 6 mg, nicotinic acid 20 mg, pyridoxine hydrochloride 3 mg, calcium pantothenate 30 mg, choline chloride 2 gm, p-aminobenzoic acid 50 mg, biotin 0.1 mg, folic acid 0.25 mg, and inositol 1 gm. Each animal received weekly 3 drops of Abbott Haliver oil U.S.P. (Haliver oil contains 60,000 U.S.P. units of vitamin A and 600 U.S.P. units of vitamin D per gm.) The above rations contain all the known members of the vitamin B complex, added in crystalline form.

Each animal was housed in an individual mesh-bottomed cage and was weighed weekly. All animals were given food and water *ad libitum*.

The composition of the rations used is given in table 1. Supplements were added to the basal as per cent of the basal, unless otherwise indicated. In all experiments both

negative and positive controls were included. The negative control received the purified ration plus desiccated thyroid, while the positive control received the purified ration alone.

After preliminary orienting experiments, it was found that a 4-week growth period could be used as an assay of the antithyrotoxic properties of a material. This assay was used in these studies.

TABLE 2

Effect on growth and survival of graded amounts of ingested thyroid substance.

LOT NO.[1]	RATION	THYROID CONTENT	AVERAGE SURVIVAL	NO. ALIVE AFTER 84 DAYS	AVERAGE WEIGHT AT		
					4 weeks	8 weeks	12 weeks
		% added[2]	days[3]		gm	gm	gm
13	A	0.0	84	3	181 ± 19	275 ± 21	302 ± 33
14	A	0.1	81	2	140 ± 18	195 ± 17	172 ± 16(2)
15	A	0.25	38	0	98 ± 8	....	....
16	A	0.5	35	0	72 ± 3(2)[4]	....	....
17	B	0.0	84	3	162 ± 27	256 ± 48	300 ± 60
18	B	0.1	84	3	154 ± 21	198 ± 8	207 ± 15
19	B	0.25	71	2	129 ± 11	179 ± 3(2)	186 ± 7(2)
20	B	0.5	39	0	109 ± 16	....	....

[1] Three rats per lot.

[2] The desiccated thyroid preparation used in the above experiment was Parke-Davis Desiccated Thyroid gland. It was 50% stronger than U.S.P. and contained 0.3% iodine in organic combination.

[3] The experiment was terminated after 84 days. Where any of the animals survived the full length of the experimental period, 84 days was averaged into the survival to obtain the *average survival.*

[4] Numbers in parentheses indicate the number of animals still alive at that time.

RESULTS

In preliminary experiments it was found that the levels of desiccated thyroid fed by Ershoff and Hershberg ('45) were too high for the strain of rats used in these studies. This may also have been due to sex differences. On dietary levels of 0.5 and 1.0% desiccated thyroid, no protective effect of yeast could be demonstrated.

Table 2 summarizes the experiment performed to determine the optimal level of desiccated thyroid at which changes in growth and survival due to yeast administration would be

most marked. Desiccated thyroid was fed at levels of 0.0, 0.1, 0.25, and 0.5% added to the purified (ration A) and yeast (ration B) rations. The data (table 2) indicate that there was a graded response to the various levels of thyroid fed. This especially can be seen for the growth depression and is also reflected in the survival data. A comparison of lots 13–16 with lots 17–20 clearly shows that yeast exerted a marked antithyrotoxic effect. The differences in growth and survival resulting from yeast feeding were most marked on a level of 0.25% desiccated thyroid. Therefore this level was used in all subsequent experiments. It can also be seen that a ration (A), containing all the known B vitamins at levels which were much higher than adequate for normal animals, was incapable of supporting a good rate of growth when supplemented with thyroid.

Other possible antithyrotoxic materials

The efficacy of a number of other materials in exerting an antithyrotoxic effect was determined. Since there is no agreement in the literature (Drill and Overman, '42; Abelin, '45, '46; Ershoff and Hershberg, '45) concerning the effect of pantothenic acid on thyrotoxicosis, the action of very large quantities of this vitamin was determined. Two groups of rats (table 3, experiment A) were fed the purified ration and a low and high level, respectively, of calcium pantothenate. The animals receiving 330 mg of the vitamin per kg of ration showed no significant improvement in growth or survival when compared to those receiving 30 mg per kg of ration. It may be concluded that an *augmented* intake of calcium pantothenate exerted no beneficial effect in these experiments.

Alopecia has been shown to be a symptom of inositol deficiency in mice (Woolley, '41) and rats (Cunha et al., '43). The reported occurrence of alopecia during the course of thyrotoxicosis (Ershoff and Hershberg, '45) indicated a possible increased requirement of inositol for rats receiving desiccated thyroid. Lots 22 and 24 (table 3, experiment A)

demonstrated that the addition of 2% inositol to the thyroid-containing ration exerted no antithyrotoxic effect.

In attempts to find other sources of the antithyrotoxic factor(s), dried whole liver powder,[2] yeast nucleic acid, and 1:20 liver powder,[2] were fed. A comparison of lots 22 and 25 shows that the dried liver preparation fed at 5% of the ration is an excellent source of the antithyrotoxic factor(s); an average weight difference of 62 gm was noted at the end of 4 weeks. The beneficial effect of liver was exhibited throughout an 11-week period while all of the rats in lot 22 died after 6 to 8 weeks.

Dried whole liver, when added at a 5% level, exerted a greater beneficial effect than did 12% dried brewer's yeast. This marked protection, by dried whole liver powder, was reflected in both growth and survival of the animals. Dried whole liver powder was also found to be active when fed at a 3% level (experiments C and D, table 3).

The addition of 5 gm of liver powder 1:20 [2] (roughly equivalent to 30 gm of dried whole liver powder) per kg of ration had a marked protective effect as is clearly shown by a comparison of lots 38 and 40. The effect of yeast nucleic acid was determined since this substance occurs in high concentration in dried yeast. It can be seen from the data in table 3 (experiment B) that the addition of 0.5% yeast nucleic acid had no beneficial effect.

A comparison of lot 28 with lot 35 indicates that yeast still exerted some antithyrotoxic action when added to the ration at a level of 5%.

Stability of the antithyrotoxic activity of liver

In order to determine the stability of the antithyrotoxic material(s) in liver, dried whole liver powder was subjected to the rather drastic treatments described below. The dry powder was suspended in 2–3 times its weight of distilled water and then divided into 3 portions and treated as follows:

[2] Wilson.

One sample was autoclaved for 1 hour at 15 pounds. The other 2 were adjusted to pH 1.0 and 10.0 with hydrochloric acid and sodium hydroxide, respectively, and then autoclaved for 1 hour at 15 pounds. These were then neutralized with sodium hydroxide and hydrochloric acid. Each of the 3 preparations was dried and ground to a fine powder before use.

The treated preparations were fed at levels equivalent to 5% whole liver powder in 1 experiment (B) and at a 3% level in another (D). The data in table 3 indicate that, at either of the levels fed in these studies, none of the above treatments resulted in a loss of the antithyrotoxic activity of the whole liver powder.

In a subsequent experiment an extract of dried whole liver powder which had antithyrotoxic activity was heated at 100°C. for 12 hours with no loss of activity. It can be concluded from the above experiments that the activity of dried whole liver powder as an antithyrotoxic agent is not destroyed by heating in water, acid, or alkaline solutions.

DISCUSSION

The data presented clearly indicate that there is present in liver a factor(s) which is essential for the *growth* of rats suffering from experimentally induced hyperthyroidism. When the ration contained all known members of the vitamin B complex, at levels much higher than accepted adequate levels, thyrotoxicity was severe; also, further addition of calcium pantothenate and inositol gave no protection, whereas the various liver preparations exerted antithyrotoxic effects. In other experiments, too detailed to describe in this paper, doubling the vitamin levels described in table 1 had no noticeable effect on growth and survival.

It may well be that thyroid feeding *increases the requirement* of the growing animal for some as yet unknown factor(s) which the animal ordinarily obtains in adequate quantities through tissue or intestinal synthesis. Another possibility which must be considered is that thyroid feeding results in *decreased synthesis* of some factor(s) essential for growth.

TABLE 3

Sources and stability of the antithyrotoxic factor(s).

LOT NO.[1]	RATION	THYROID SUBSTANCE [2]	SUPPLEMENT	AVERAGE WEIGHT 4 WEEKS
		% added		*gm*
			Experiment A	
21	A	0.0	None	154 ± 10
22	A	0.25	None	81 ± 16
23	A	0.25	300 mg calcium pantothenate per kg of ration	65 ± 15
24	A	0.25	2% inositol	78 ± 16
25	A	0.25	5% dried whole liver substance [3]	143 ± 11
26	B	0.25	None	124 ± 13
			Experiment B	
27a	A	0.0	None	159 ± 2
27b	B	0.0	None	177 ± 6
28	A	0.25	None	103 ± 1
29	A	0.0	5% dried whole liver powder	195 ± 9
30	A	0.25	5% dried whole liver powder	139 ± 15
31	A	0.25	5% autoclaved whole liver powder	159 ± 6
32	A	0.25	5% whole liver powder autoclaved in acid solution	147 ± 15
33	A	0.25	5% whole liver powder autoclaved in alkaline solution	147 ± 11
34	B	0.25	None	141 ± 10
35	A	0.25	5% dried brewer's yeast	135 ± 13
36	A	0.25	0.5% yeast nucleic acid	94 ± 12
			Experiment C	
37	A	0.0	None	189 ± 9
38	A	0.38	None	119 ± 4
39a	A	0.0	3% dried whole liver powder	190 ± 10
39	A	0.38	3% dried whole liver powder	141 ± 11
40	A	0.38	5 gm liver powder 1: 20 per kg of ration [4]	142 ± 10
			Experiment D	
52	A	0.0	None	179 ± 14
53	A	0.38	None	129 ± 3
59	A	0.38	3% whole liver powder autoclaved in alkaline solution	163 ± 8
60	A	0.38	3% dried whole liver powder	142 ± 13

[1] Four rats per lot.

[2] The desiccated thyroid used in experiments A and B of the table was a Parke-Davis (0.3% iodine) preparation; in C and D, Wilson desiccated thyroid U.S.P. (0.2% iodine) was used. The substitution was made on an equi-iodine basis.

[3] Wilson.

[4] Liver powder 1: 20 (Wilson) is prepared by water extraction of whole liver. One gm is equivalent to 20 gm of fresh liver.

Still a third alternative is that liver and yeast contain a substance which directly antagonizes the action of the thyroid hormone. Further work is necessary to determine how the beneficial effect of liver in counteracting thyrotoxicity is exerted.

The antithyrotoxic factor(s), as can be seen from the data, is not destroyed by heating in neutral, acid, or alkaline suspension. Its presence in liver powder 1:20 [3] indicates it to be water soluble. The factor(s) reported in this paper does not appear to be identical with the monkey anti-anemia factor (also present in liver) reported recently (Ruegamer et al., '47) since the latter factor is heat labile.

While this paper was in preparation, Ershoff ('47) reported the presence of a factor in liver, other than the known B vitamins, which was necessary for the growth of immature rats fed desiccated thyroid. The results reported in this paper are in agreement with his data. Work is now in progress on the fractionation of liver preparations in order to determine the properties of, and to purify the active material observed in these studies. Progress which has been made in that direction will be reported in a later paper.

SUMMARY

1. The presence in yeast of an antithyrotoxic factor, which has been previously reported, has been confirmed in these studies.

2. Liver has been found to be a better source of the antithyrotoxic material than yeast. This factor is not one of the known B vitamins. It is heat stable and under the experimental conditions employed is not destroyed by autoclaving in acid or alkaline solution.

3. Calcium pantothenate and inositol did not exert an antithyrotoxic effect when fed at very high levels in a purified ration.

[3] Wilson.

ACKNOWLEDGMENTS

We are indebted to Abbott Laboratories, North Chicago, Ill., for Haliver Oil; to Merck and Co., Rahway, N. J., for crystalline vitamins; to the Wilson Laboratories, Chicago, Ill., for liver powder 1:20, dried whole liver powder and desiccated thyroid U.S.P.; to the Lederle Laboratories, Inc., Pearl River, N. Y., for synthetic folic acid.

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THE EFFECT OF THE INGESTION OF HIGH LEVELS OF RIBOFLAVIN ON THE AMOUNT IN THE MILK AND URINE[1]

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(Received for publication June 23, 1947)

During the course of studies on the effect of the ingestion of synthetic thiamine, riboflavin, nicotinic acid and pantothenic acid on the amounts of these vitamins in the milk of the cow it was observed that by the microbiological method the riboflavin content of the milk from cows fed the synthetic vitamins showed varying degrees of increase from none up to a maximum of a 2-fold increase. Usually the milk showed a very distinct greenish-yellow color, characteristic of aqueous solutions of riboflavin. Samples of milk from cows fed rather large amounts of riboflavin, when measured by the standard fluorometric procedure, gave values approximately 5 times higher than the values obtained microbiologically.

This observation suggested that at least in certain species the apparent riboflavin value of the milk following the ingestion of riboflavin might differ by as much as 500%. This posed a problem of both applied importance and possibly one of comparative biochemistry of a species difference in metabolism of riboflavin. Furthermore it seemed important to determine by feeding experiments with rats which of the 2 assay

[1] The authors are indebted to Merck and Company for the riboflavin, to J. A. Gray for providing the sheep used in these investigations, and to Patricia G. Sparks and Helen Keene for technical assistance.

procedures measured the true biological activity of the riboflavin in the milk.

EXPERIMENTAL

In these studies milch goats and sheep were used since metabolism cages were available permitting the quantitative collection of the urine simultaneously with the collection of the milk. With the goats the full amount of milk secreted was withdrawn from the udder, but with ewes it was not possible to obtain all of the milk. There was also a marked difference in the amount of milk produced by the goats, and for these reasons the data are more uniform and consistent when expressed on a ml basis rather than on a 24-hour basis.

The urine and milk were collected for 2 consecutive days. Two gm of synthetic riboflavin were then administered in a gelatin capsule for each of 3 consecutive days. The urine and milk were collected on the second and third day of ingestion of the riboflavin. Precautions were taken to prevent destruction of the riboflavin in the milk and urine by photolysis.

The urine and milk collected both before and after the ingestion of riboflavin were assayed by the standard microbiological method of Snell and Strong ('39). Riboflavin was also measured by the fluorometric procedure with a Coleman photofluorometer after 3 different treatments of the samples. The milk was autoclaved with 0.1 N HCl for 30 minutes. After adjusting the pH to about 4.6 the aliquot was made up to volume and filtered. The urine was merely adjusted to the proper pH and diluted. A sufficiently large sample was prepared so that all assays could be made from the initial preparation.

The milk and urine samples for the microbiological assays received no treatment other than that previously mentioned. The riboflavin was also measured fluorometrically for the milk and urine samples so prepared without any attempt to remove non-riboflavin fluorescent compounds. This value is referred to in the tables as "untreated" under fluorometric. The second fluorometric procedure consisted in treating the

diluted urine or filtrate from the milk with potassium permanganate and hydrogen peroxide (Scott et al., '46). The values obtained by this treatment are referred to in the tables under the column headed "$KMnO_4$." This fraction was then adsorbed on florisil and eluted with a 20% pyridine-2% acetic acid solution. The values obtained by this treatment are referred to in the tables under the column headed "$KMnO_4$ plus florisil."

RESULTS AND DISCUSSION

Milk

The data on the riboflavin values for milk obtained by the different analytical procedures are presented in table 1. Since the goat and sheep responded in a similar manner to the

TABLE 1

Apparent riboflavin in goat's and ewe's milk. (Values in μg per ml.)

ANIMAL[1]	DIETARY TREATMENT	MICRO-BIOLOGICAL	FLUOROMETRIC		
			Untreated	$KMnO_4$	$KMnO_4$ plus florisil
Goat no. 1	Basal	3.7	4.0	3.8	3.9
Goat no. 1	Riboflavin	4.3	19.8	20.0	19.8
Goat no. 2	Basal	3.4	3.7	3.7	3.6
Goat no. 2	Riboflavin	3.5	25.7	25.2	24.0
Goat no. 3	Basal	2.4	2.6	2.5	2.5
Goat no. 3	Riboflavin	3.7	14.7	14.3	12.0
Sheep no. 1	Basal	5.7	6.9	6.3	5.3
Sheep no. 1	Riboflavin	6.7	44.8	45.0	34.2
Sheep no. 2	Basal	5.4	6.3	5.6	5.2
Sheep no. 2	Riboflavin	7.8	37.6	36.6	26.2

[1] The weights of the animals starting with goat no. 1 and proceeding down the table were 50.0 kg, 43.6 kg, 47.7 kg, 54.5 kg and 50.9 kg.

ingestion of riboflavin and have similar values for the milk, the data for the 2 species are considered together.

The fluorometric values on the untreated normal milk average about 15% higher than the microbiological values. This indicates that goat's and ewe's milk contain some non-ribo-

flavin fluorescent compounds. Treating with potassium permanganate and hydrogen peroxide gave values slightly lower than the untreated values. The microbiological values agreed very well with the values obtained after treating with $KMnO_4$ and adsorbing on florisil. The average riboflavin content of the 5 samples of normal milk by the microbiological method was 4.12 μg per ml as compared to 4.10 μg per ml by the fluorometric method following treatment with $KMnO_4$ and adsorbing on florisil.

Following the ingestion of 2 gm of riboflavin daily there was an increase of only 26% in the riboflavin content of the milk as measured by the microbiological method. The average riboflavin content of the milk from the animals on the basal diet was 4.12 μg per ml as compared to 5.20 μg per ml following the ingestion of riboflavin. The magnitude of the increase is very small considering the amount of riboflavin ingested. These observations differ from the findings with women where there appears to be a more direct correlation between the intake of riboflavin and the concentration in the milk (Neuweiler, '37).

Following the ingestion of 2 gm daily of synthetic riboflavin there was a marked increase in the riboflavin value of the milk of all animals as measured fluorometrically. Using the "$KMnO_4$ plus florisil" procedure the riboflavin values were 5 to 6 times that obtained by the microbiological procedure. Obviously 1 of these methods represents an error probably due to fluorescent compounds measured by the fluorometric procedure which do not have biological activity. The validity of this assumption was tested by feeding experiments with rats.

Rat growth

Milk from 2 goats and 1 ewe was fed to groups of rats and the growth response used as a measure of the true riboflavin present. Milk collected during the period the basal diet was fed and during the period of riboflavin ingestion was evaporated on purified diets at levels to provide the same amount

of riboflavin in the diets when measured by the microbiological procedure. The diet used for measuring the biological activity of the riboflavin in the milk was the same as that previously described (Schweigert and Pearson, '47) except for the riboflavin. On the basis of the fluorometric value the diet containing the riboflavin milk provided 150 μg or more of riboflavin per 100 gm of diet. This is adequate to permit maximum rates of gain by the rat. That the diet was adequate in all factors

TABLE 2

Growth of rats supplemented with milk as source of riboflavin.

SOURCE OF RIBOFLAVIN	μG RIBOFLAVIN/100 GM DIET		BODY WEIGHT [1]		
	Microbiological	$KMnO_4$ plus florisil	Initial	Final	Gain per week
		Goat's milk	*gm*	*gm*	*gm*
Normal milk 7 ml/100 gm diet	23.4	25.3	34.6	52.0	5.8
Riboflavin milk 7 ml/100 gm diet	24.1	179.2	39.0	53.5	4.8
Synthetic riboflavin	150.0	150.0	41.2	103.0	20.6
		Ewe's milk			
Normal milk 5.1 ml/100 gm diet	29.3	27.1	42.3	60.6	6.1
Riboflavin milk 4.4 ml/100 gm diet	29.3	150.0	42.8	59.1	5.4
Synthetic riboflavin	150.0	150.0	42.2	89.5	15.8

[1] Data are averages. There were 6 rats in each group and the length of the feeding period was 3 weeks.

except riboflavin is apparent from the good gains made by the rats fed the diet supplemented with 150 μg of synthetic riboflavin per 100 gm of diet. The results for the milk from the 2 goats were in essential agreement. Therefore, to conserve space the growth data for milk from only goat no. 2 and ewe no. 1 are presented in table 2.

The rats fed the milk from the goat on the basal diet and the milk collected during the period of riboflavin ingestion

made essentially the same rate of gain. The average weekly gain of the rats receiving the diet containing the milk collected during the basal period was 5.8 gm while the rats fed the diet containing the riboflavin milk gained an average of 4.8 gm per week. The diets containing the ewe's milk each provided 29.3 μg riboflavin per 100 gm according to the microbiological assay. By the fluorometric method the diet containing the riboflavin milk should have provided 179.2 μg of riboflavin per 100 gm. The average weekly gains for the 2 groups of rats were 6.1 and 5.4 gm, respectively, for the rats receiving the diet containing the normal milk and the riboflavin milk. From these data it is clear that the riboflavin measured fluorometrically above that measured microbiologically is not active for the rat.

It is of interest that the rats receiving the milk collected during the period of riboflavin feeding made slightly lower gains than the rats receiving the diets containing the milk from the animals on the basal diet. The average weekly gain by the rats fed the normal milk from the second goat (not shown in table 2) was 6.4 gm as compared with 4.8 gm by the rats fed the diet containing the milk collected during the period of riboflavin feeding. While the data here are meager, the possibility that metabolites formed from riboflavin by the goat and sheep may have a depressing effect on growth of rats should not be overlooked.

Urine

The ingestion of riboflavin resulted in an increase of around 3-fold as measured microbiologically in the level of riboflavin in the urine of the goats. The increase was of a smaller order for the ewes. The volume of urine voided by ewe no. 1 during the period of riboflavin ingestion was 940 ml per 24 hours as compared to 730 ml during the basal period. The daily excretion of riboflavin by this animal was greater during the period of riboflavin feeding than during the basal period, but it does not show up on a ml basis due to the difference in volume of urine.

The microbiological values on the urines collected during the basal period are in good agreement with the values obtained by the fluorometric procedure after treating with $KMnO_4$ and adsorbing on florisil.

On the basis of microbiological assay the maximum recovery in the urine of the 2 gm ingested was 4.8 mg. The average recovery in the urine of ingested riboflavin was 4.0 mg, 4.8 and 2.9 mg, respectively, for goats 1, 2 and 3. The corresponding recoveries for the ewes were 1.0 and 2.8 mg for numbers

TABLE 3

Apparent riboflavin in the urine of sheep and goats. (Values in μg per ml.)

ANIMAL[1]	DIETARY TREATMENT	MICRO-BIOLOGICAL	FLUOROMETRIC		
			Untreated	$KMnO_4$	$KMnO_4$ plus florisil
Goat no. 1	Basal	4.3	5.1	5.0	4.6
Goat no. 1	Riboflavin	15.3	156.0	160.0	107.0
Goat no. 2	Basal	2.9	5.0	5.0	3.6
Goat no. 2	Riboflavin	7.8	164.0	143.8	124.0
Goat no. 3	Basal	4.0	6.1	6.1	4.5
Goat no. 3	Riboflavin	12.6	147.0	135.6	136.0
Sheep no. 1	Basal	7.2	7.0	6.9	6.8
Sheep no. 1	Riboflavin	6.7	138.4	124.0	110.0
Sheep no. 2	Basal	2.8	3.7	3.7	2.7
Sheep no. 2	Riboflavin	5.6	98.0	87.0	73.0

[1] The weights of the animals starting with goat no. 1 and proceeding down the table were 50.0 kg, 43.6 kg, 47.7 kg, 54.5 kg and 50.9 kg.

1 and 2. These data suggest that the riboflavin is rapidly metabolized by the sheep and goat to compounds without biological activity.

Following the ingestion of riboflavin the values obtained by the fluorometric procedure following treatment with $KMnO_4$ and adsorbing on florisil were from 7 to 16 times the values obtained by the microbiological assay. Adsorbing on florisil following treatment with $KMnO_4$ was more effective in removing non-riboflavin fluorescent pigments than the $KMnO_4$ treatment only. From these data it is apparent that

when goats and sheep are fed large amounts of riboflavin, compounds having fluorescent properties but not biological activity are formed. The non-riboflavin fluorescent compound is secreted into the milk and excreted by the renal pathway.

The fluorescent compound or compounds without biological activity that are formed by the goat and sheep from ingested riboflavin have not been identified. It is possible that the ribose moiety of the riboflavin molecule is broken off and that 6 : 7 dimethyl-alloxazine or lumichrome is produced. This compound is biologically inactive, but it is highly fluorescent. Whether the site of formation of the biologically inactive compound is in the rumen or in the tissues following absorption remains to be determined.

Effect of ingestion of large amounts of riboflavin by rats

From a comparative standpoint it was of interest to determine whether or not the rat when given large amounts of riboflavin would excrete non-riboflavin fluorescent compounds similar to the goat and sheep.

For these studies 3 mature rats were used for each level of riboflavin administered. The urine was collected for 2 consecutive days. Ten mg of riboflavin were administered by stomach tube on each of 3 consecutive days, and the urine collected on the second and third days. The urines were assayed for riboflavin by the microbiological method and by each of the 3 fluorometric procedures. Of the latter only the value obtained by treating with $KMnO_4$ and adsorbing on florisil will be referred to here. The 10 mg of riboflavin on the basis of body weight, was approximately equivalent to the level ingested by the goats and sheep.

On the basal diet the average daily urinary excretion of riboflavin as measured by the microbiological procedure was 15.9 μg as compared with 14.7 μg by the fluorometric procedure. Following the ingestion of 10 mg of riboflavin the average daily urinary excretion measured by the microbiological procedure was 114.3 μg as compared to a value of 137.0 μg by the

fluorometric method. To another group of 3 rats 20 mg of riboflavin were administered daily. This is about double the amount ingested by the sheep and goats per kg of body weight. On the basal diet the average daily riboflavin excretion was 16.3 μg and 19.4 μg as measured by the microbiological and fluorometric methods, respectively. The corresponding figures during the period of riboflavin ingestion were 208.4 and 223.4 μg of urinary riboflavin. The magnitude of the differences between the biological values and the fluorometric values is essentially the same for the urine from the rats on the basal diets as for the rats receiving the riboflavin. From these data it is apparent that the sheep and goat differ from the rat in their metabolism of riboflavin in that the rat does not form appreciable amounts of biologically inactive compounds that are measured by the fluorometric procedures for riboflavin.

Observations on human beings

The question whether man responds in a manner similar to the rat or the goat and sheep on the ingestion of relatively large amounts of riboflavin is of considerable interest. Should human beings metabolize ingested riboflavin in a manner similar to the goat and sheep, data on excretion studies may differ considerably depending on whether the analytical data were obtained by the microbiological method or fluorometric method. On the other hand, if man metabolizes ingested synthetic riboflavin in a manner similar to the rat, it would make no essential difference whether the riboflavin determinations were made by the fluorometric method or by the microbiological method.

For these studies 2 male adults were used. The urine was collected quantitatively for 2 days. Twenty mg of riboflavin in the form of tablets were ingested daily for the 3 succeeding days. The urine was collected on the second and third days of the period. The level of riboflavin intake was about 10 times the daily allowance recommended by the Food and Nutrition Board ('45).

There was no significant difference in the riboflavin values obtained by the microbiological assay and the fluorometric method where the urine was treated with $KMnO_4$ and adsorbed on florisil. Following the ingestion of riboflavin the urine of subject no. 1 contained 6.28 μg of riboflavin per ml as measured microbiologically and 6.47 μg by the fluorometric method. The corresponding values for the urine of subject no. 2 were 4.50 μg and 3.37 μg per ml by the microbiological and fluorometric procedures, respectively.

From these data it is apparent that at the levels of riboflavin that would normally be ingested by human beings, that comparable results for riboflavin in the urine could be expected using either the microbiological or standard fluorometric procedures.

SUMMARY

The oral administration of large amounts of riboflavin to goats and sheep increased the true riboflavin content of the milk by only about 26%. Measured by the fluorometric procedure the riboflavin values were for the milk approximately 5 times greater than the values obtained by the microbiological method and for the urine the fluorometric values were about 12 times higher. The riboflavin content of the milk as measured by the microbiological assay agreed well with the growth response of rats fed the milk as a source of riboflavin.

The microbiological and fluorometric values for the riboflavin content of the urine of rats were in good agreement even when massive doses of 10 or 20 mg of riboflavin were administered daily. The microbiological and fluorometric values were in good agreement for urine from humans that had been fed 20 mg of riboflavin per day.

Goats and sheep fed large amounts of riboflavin evidently form a fluorescent compound that is measured by the fluorometric procedure for riboflavin. The unidentified compound secreted into the milk and excreted by the renal pathway is not biologically active.

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SOME STUDIES ON THE NUTRITIVE VALUE OF BUTTER FATTY ACIDS[1]

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(Received for publication May 24, 1947)

The effect of volatile fatty acids on the weight gain of young rats was compared with that of other fatty acid fractions of butter fat by Schantz et al. ('40) and Brown and Bloor ('45). While both investigations indicate a dietary inferiority of the short-chain fatty acids, their effect as part of butter fat is still doubtful. Schantz et al. fed them mixed with corn oil and liquid skim milk, a diet which admittedly produces diarrhea (Hart, '45), whereas Brown and Bloor gave them as part of a Sherman B diet. The latter workers also observed a laxative action and reduced the fatty acid content of the volatile fatty acid diet from the 10% level of the control diet to 6%. In the presence of lactose the saturated fatty acids of butter were found to be superior to the unsaturated fatty acids by Schantz et al., but not by Brown and Bloor. Considering the work of Henry et al. ('45), Henderson et al. ('45), Jack et al. ('45), and particularly the recent discovery of the growth-promoting effect of vaccenic ($\triangle^{11, 12}$ — octadecenoic) acid in summer butter (Boer et al., '47), one would expect the unsaturated rather than the saturated fatty acid fraction of butter to be more effective in promoting the weight gain of young rats. The effect of

[1] From a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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unsaturation as such is by no means clearly established, since the fatty acid composition of butter underwent some alteration in the feeding experiments thus far reported. The amount and concentration of vitamin A stored in the livers of rats given raw carrots has also been reported to vary with the nature of the dietary fatty acids (Brown and Bloor, '45). On an average, rats fed unsaturated fatty acids stored more and higher concentrations of vitamin A than those on a saturated fatty acid diet. Whether this effect operates on the conversion of carotene to vitamin A can be elucidated by feeding preformed vitamin A instead of carotene.

EXPERIMENT 1

Three groups of rats were given, respectively, butter fatty acids, their non-volatile portion, and hydrogenated butter fatty acids. The fatty acids of butter were prepared as follows: 5 pounds of butter, bought on the local market, were melted on the steam-bath and then allowed to solidify in a beaker in the ice-chest. The aqueous phase was drained off after puncturing the fat cake. The butter fat was washed with water in the molten state and again separated from water as before. This was repeated once more. Saponification was carried out by refluxing the fat on a steam-bath for 2 hours with 8 liters of 10% KOH in 95% methanol in a round bottom flask. At the end of this period the flask was converted into a continuous liquid extractor of the type described by Hilditch ('40). The extraction of the non-saponifiable matter with low-boiling petroleum ether was continued until the soaps gave a negative Carr-Price test. This took about 10 hours. The soap solution was then transferred to a 5-gallon bottle, cooled by adding chopped ice, and acidified to Congo Red paper with HCl. Ether was added, the bottle stoppered and laid on its side. To mix its contents the bottle was rolled back and forth, then put in an upright position and allowed to stand until the ether separated out. The ether solution of fatty acids was syphoned off into another 5-gallon bottle. After repeated extraction of the fatty acids with ether, the com-

bined ether extracts were washed with a small amount of water to remove most of the alcohol, then dried overnight with calcium chloride. The solvent was removed from the fatty acids by vacuum distillation. This resulted in 1220 gm of fatty acids with an average molecular weight (determined by titration) of 251.0 and an iodine value (Wijs' method) of 59.1. The product was divided into 3 portions.

A 400-gm portion of butter fatty acids was steam distilled for 5½ hours, at which time about 5 liters of distillate had collected in the receiver. The residue was allowed to solidify in the ice-box. The cake of fatty acids was separated from the water phase and dissolved in petroleum ether. To this solution a petroleum ether extract of the water phase was added. After drying and evaporation as before, 361 gm of non-volatile fatty acids remained. Their average molecular weight was 264.4.

Another 400-gm portion of the original fatty acids was introduced into a high pressure hydrogenation bomb in the dry, molten state, together with about 5 gm Raney nickel catalyst. The hydrogenation was carried out in the absence of solvents at 2200 lbs. and 150°C. with agitation for 2 hours. To remove the catalyst the hydrogenated fatty acids were dissolved in petroleum ether and shaken with fairly concentrated hydrochloric acid. The petroleum ether solution was then washed with water, dried, and evaporated. The iodine value of the hydrogenated product was 40.6.

The diets used were similar to those employed by Brown and Bloor ('45). The Sherman B diet was modified in such a way that the fatty acids under investigation constituted the fat component to the extent of 9.9% of the diet. Three rations were prepared containing, respectively, the original butter fatty acids, the non-volatile fatty acids, and the partially hydrogenated fatty acids. Diets were prepared in 1 kg batches by kneading the molten fatty acids into the mixture of whole wheat flour, skim milk powder, and salt. Rats were fed as much as they would consume within a 2-day period, the remainder of the food being stored in the ice-box.

Thirty albino rats of the Wistar strain, 15 male and 15 female, 23–24 days old, were distributed as equally as possible with respect to sex, age, litter, and weight over the 3 experimental groups. Although no special precautions were taken to prevent rats from spilling their food, the use of heavy, deep cups, only half full of diet, permitted a good rough estimate of the relative weekly food consumption per group of males and females, because the amount of food spilled was small and about equal. The rats were weighed weekly and their gains recorded.

Desynon,[3] a solution containing 500 U.S.P. units vitamin A and 50 U.S.P. units vitamin D_3 in 90% alcohol per drop, was used as a source of vitamins A and D. The solution was diluted 5 times with milk. Each rat received a drop of this preparation, i.e., 100 units vitamin A and 10 units vitamin D on the day the experiment was started and every week thereafter, except on the last day of the experiment. Solutions of Desynon in milk were prepared immediately before use and fed by means of the special dropper supplied with the product. Due to gradual deterioration the vitamin A content of Desynon fell to such an extent that the animals received only about half of the initial dose by the end of the experiment. At the end of the fifth week the rats were killed by illuminating gas, and their livers examined for vitamin A by the methods of McCoord and Luce-Clausen ('34) and Clausen et al. ('42). No appreciable amounts of vitamin A could be detected.

Table 1 summarizes the results of the first experiment. The mean weight gains since the start of the experiment together with their standard errors, the weekly food consumption, and the efficiency of food conversion, expressed as the percentage by weight of food converted into body substance are given for each week. The effect of volatile fatty acids on weight gain was evaluated by calculating Fisher's "t" for the difference between the mean weight gains of male and female rats on the diet of the original and of the non-volatile fatty acids.

[3] Winthrop Chemical Co., Inc.

This gave the following "t" values for the 5 consecutive weeks of the experiment: Males: 1.1, 1.1, 2.0, 1.5, 1.4; Females: 0.6, 0.5, 0.3, 1.4, 1.6. The corresponding probability values, taken from Fisher's table (Fisher, '38) are: Males: 0.3, 0.3, 0.1–0.05, 0.2–0.1, 0.2–0.1; Females: 0.6–0.5, 0.6, 0.8–0.7, 0.2 0.2–0.1. Regarding any probability level below the conventional 0.05 as significant, it is evident that there is no significant difference between the weight gain of males or females

TABLE 1

Weight gains, food consumption, and efficiency of food conversion of rats on diets containing various butter fatty acids.

	WEEK	NON-VOLATILE FATTY ACIDS		ORIGINAL FATTY ACIDS		PARTLY HYDROGENATED FATTY ACIDS	
		♂	♀	♂	♀	♂	♀
Cumulative mean weight gains in gm	1	22 ± 3	18 ± 2	24 ± 3	17 ± 3	11 ± 2	17 ± 2
	2	46 ± 3	39 ± 3	48 ± 2	37 ± 7	37 ± 6	39 ± 7
	3	70 ± 4	59 ± 7	75 ± 3	57 ± 10	54 ± 10	57 ± 5
	4	94 ± 2	75 ± 7	98 ± 5	67 ± 8	69 ± 17	73 ± 4
	5	20 ± 3	89 ± 8	124 ± 5	80 ± 8	88 ± 26	89 ± 2
Mean weekly food consumption per rat, gm	1	45	45	51	44	44	48
	2	62	61	66	56	60	58
	3	70	64	72	62	61	63
	4	75	68	74	60	62	63
	5	82	71	84	60	68	63
Mean efficiency of food conversion	1	49	40	47	39	25	35
	2	39	34	36	36	43	38
	3	34	31	38	32	28	29
	4	32	24	31	17	24	25
	5	32	20	31	22	28	25

fed the original butter fatty acids and those fed the non-volatile portion of these acids.

The effect of hydrogenation of the dietary fatty acids on weight gain was similarly evaluated by calculation of Fisher's "t" for the difference between the mean weight gains of males and females on the original and on the hydrogenated fatty acid diet. The following "t" values were obtained: Males: 7.8, 3.5, 4.1, 3.2, 2.8; Females: 0, 0.4, 0, 1.3, 2.1. The correspond-

ing probability values are: Males: < 0.01, < 0.01, < 0.01, 0.02–0.01, 0.02; Females: > 0.9, > 0.9, > 0.9, 0.3–0.2, 0.1–0.05. This means that the weight gain of males, but not of females, is significantly lower on a diet containing partially hydrogenated fatty acids than on the original butter fatty acid diet.

The figures on food consumption and conversion indicate that the efficiency usually falls off with time, and that it is usually lower for females than for males of the same group. Some, though inconsistent, differences appear between the males fed partially hydrogenated and the original butter fatty acids. Hydrogenation seems to have reduced the food utilization of the males, sometimes even below that of the females of the same group.

EXPERIMENT 2

In order to determine the effect of complete hydrogenation of the dietary butter fatty acids on weight gain and vitamin A storage a similar experiment was set up with completely hydrogenated fatty acids and larger vitamin supplements. The method of preparation and purification of the fatty acids of butter was the same as that described under experiment 1. About half of the fatty acids were hydrogenated at 1950 lbs. and 150°C. in the presence of about 7 gm Raney nickel and 100 ml absolute alcohol with agitation for 8 hours. This time the iodine value of the fatty acids dropped to 1.1 after hydrogenation. Two diets were prepared, 1 containing the original fatty acids of butter, the other their hydrogenated counterpart. Twenty Wistar rats, 10 male and 10 female, all 29 days old, were evenly distributed over the 2 groups. The Desynon solution was analyzed weekly for vitamin A by evaporating an aliquot portion of a drop in a colorimeter tube, dissolving it in purified (McCoord and Luce-Clausen, '34) chloroform, and then following the procedure of Clausen et al. ('42). Instead of milk, glycerol was used to reduce the strength of the alcohol in Desynon. Mixtures of 2 parts of glycerol and 1 part of Desynon were given by dropper in such amounts that the rats received about 300 units vitamin A at the start

of the experiment and 1 week thereafter, 400 units at the end of the second week, 500 units at the end of the third week, and 600 units at the end of the fourth week. The number of units of vitamin D given was about one-tenth that of vitamin A. All rats were killed at the end of the fifth week and their livers analyzed for vitamin A by the method previously mentioned.

TABLE 2

Weight gains, food consumption, and efficiency of food conversion of rats on diets containing unchanged and completely hydrogenated butter fatty acids.

	WEEK	ORIGINAL		COMPLETELY HYDROGENATED	
		♂	♀	♂	♀
Cumulative mean weight gains, in gm	1	20 ± 4	14 ± 2	21 ± 4	16 ± 2
	2	46 ± 7	34 ± 5	46 ± 4	37 ± 4
	3	73 ± 12	53 ± 6	67 ± 4	53 ± 5
	4	98 ± 16	67 ± 2	91 ± 7	69 ± 4
	5	121 ± 21	75 ± 4	110 ± 7	77 ± 5
Mean weekly food consumption, in gm	1	55	52	64	54
	2	71	61	72	66
	3	86	67	82	71
	4	88	70	83	74
	5	100	69	87	74
Mean efficiency of food conversion	1	36	27	33	30
	2	37	33	35	32
	3	31	28	26	23
	4	28	20	29	22
	5	23	12	22	11

The results of the second experiment are summarized in tables 2 and 3. Statistical evaluation of the weight differences between rats fed the original and the completely hydrogenated butter fatty acids by Fisher's "t" test gave for males: 0.4, 0, 1.0, 0.8, 1.0, and for females: 1.4, 1.0, 0, 0.8, 0.7. Corresponding P values are, for males: 0.7, > 0.9, 0.4–0.3, 0.5–0.4, 0.4–0.3, and for females: 0.2, 0.4–0.3, > 0.9, 0.5–0.4, 0.5. Thus, there was no significant difference between the weight gain of rats receiving the original butter fatty acids and those

fed the completely hydrogenated fatty acids. The efficiency of food conversion can again be seen to fall off with time. It is smaller for females than for males, but there is no appreciable difference between the 2 groups in that respect.

Table 3 gives the mean vitamin A concentrations in I.U./100 gm of wet liver, the mean amount of vitamin A stored in the livers of each of the 2 groups of male and female rats used in experiment 2, and the standard errors of the means. Evaluation of the difference between mean vitamin A concentrations of the 2 groups by Fisher's "t" test gave 0.9 for males, and 0.8 for females. This corresponds to probability values of

TABLE 3

Vitamin A in the livers of rats on diets containing unchanged, and completely hydrogenated butter fatty acids.

	SEX	MEAN CONCENTRATION IN I.U./100 GM WET TISSUE	MEAN AMOUNT IN I.U.
Original	M	2201 ± 390	224 ± 20
	F	5823 ± 1310	403 ± 94
Completely hydrogenated	M	1858 ± 653	176 ± 55
	F	6567 ± 1290	505 ± 105

0.4 and 0.5–0.4 for males and females, respectively. Differences between mean amounts yielded "t" values of 1.6 and 1.5, and P values of 0.2–0.1 and 0.2–0.1 for males and females in the order given. Thus, there was no significant difference between the amounts or concentrations of vitamin A in the livers of either male or female rats, regardless of whether the fatty acids of butter were fed in their original or completely hydrogenated form. Females of both groups stored about twice as much vitamin A as males of both groups.

DISCUSSION

If the increased weight gain is the criterion chosen as a measure of nutritive value, the *ad libitum* method of feeding should be of greater practical value that the paired feeding

technique (Boutwell et al., '41). Food found superior by the *ad libitum* method might owe its growth-promoting effect to greater palatability or stimulation of the appetite or some other incidental properties, but in any case increased growth response will indicate a superiority in some practically relevant respect.

Steam distillation strips the butter fatty acids of those of low molecular weight, as shown by an increase in the molecular weight. By comparing rat growth on a fatty acid supplement lacking the volatile fatty acids with that on an equal quantity of the untreated fatty acids of butter the effect of the volatile fraction can be determined. The chief advantages of this method may be stated as follows: (1) No reduction in fat intake (Brown and Bloor, '45) is necessary, since such a regimen does not produce diarrhea. (2) Other fats need not be added to equalize the fat intake without increasing the proportion of volatile fatty acids (Schantz et al., '40). (3) The volatile fatty acids are diluted by the specific amount and proportion of the other naturally occurring fatty acids, increasing the practical significance of the results. The results indicate that the volatile fatty acids, as they naturally occur in butter fat, have no particular influence on growth or efficiency of food utilization. The harmful effect of these fatty acids, observed by Schantz et al. and by Brown and Bloor, must be ascribed to the absence of the other butter fatty acids from their diets. On the other hand, the more flavorful short-chain fatty acids did not appreciably increase appetite and food consumption of weanling rats. Hilditch and Jasperson ('42) found 2 changes to occur in the fatty acid composition of butter when the diet of milch cows is changed from hay to fresh grass, namely, reduction of the percentage of volatile fatty acids, and an increase in the amount of oleic acid. The growth-promoting effect of summer butter now appears to be due not to the former change, as has been suggested by them, but to the increase in the oleic acid fraction, which also contains vaccenic acid.

It seems difficult to reconcile the postulate of a growth-promoting saturated fatty acid with the finding that the unsaturated fatty acid vaccenic acid has growth-promoting activity. An attempt was made to test both contentions by feeding the hydrogenated fatty acids of butter and comparing their influence on growth with that of untreated butter fatty acids. In such an experiment unsaturation is the only variable — the composition of the fatty acid mixture remains unchanged. In the first experiment on the effect of hydrogenation on the growth-promoting value of the fatty acids of butter the iodine value of the fatty acids was lowered from 59.1 to 40.6. The growth of male rats on the partially hydrogenated fatty acid diet was definitely inferior to that of their litter-mates on untreated fatty acids, and their efficiency in converting food into body substance was somewhat reduced. In the second experiment, however, when the fatty acids were for all practical purposes completely hydrogenated, there was no difference in weight gain or in the efficiency of food conversion between animals of both sexes on hydrogenated and untreated fatty acids. Since rats grew as well on completely hydrogenated fatty acids as on the original fatty acids of an iodine value of about 60, unsaturation clearly could not have been the sole factor in determining the growth rate in experiment 1. Except for the iodine value of the hydrogenated fatty acids the diet of the animals in experiment 1 differed from that used in experiment 2 in 1 important respect. In experiment 1 each rat received weekly from 50 to 100 units of vitamin A and about 10 units of vitamin D. In the course of the whole experiment a rat could not have received more than 500 units of vitamin A and 50 units vitamin D. But the total amount of vitamin A consumed per rat in the second experiment was around 2000 units, together with about 200 units vitamin D. It is conceivable that the reduction of the unsaturated growth-factor of Boer et al. ('47), namely vaccenic acid, or of unsaturated fatty acids in general by partial hydrogenation had a deleterious effect on rat growth by lowering the efficiency of food conversion only

when the vitamin intake was relatively low, but not when a more generous vitamin supplement was given. Boer and Jansen ('42) have also observed that upon addition of vitamin D (but not A) to winter butter the difference in response between summer and winter butter disappears, while addition of vitamin D to summer butter is without effect. By the same token partial hydrogenation in experiment 1 had a greater effect when the vitamin supply in the diet was relatively scanty, than complete hydrogenation in experiment 2 where higher doses of vitamin D offset the removal of unsaturated material.

It is interesting to note that Boer and Jansen ('42) as well as Brown and Bloor ('45) used only male rats. Female rats may have the ability to synthesize unsaturated fatty acids more efficiently than males, or else they may use their supply of fat-soluble vitamins more economically, as evidenced by the greater storage of vitamin A in the livers of females. Sex differences in the metabolism of lipids or lipid-soluble vitamins are not at all unusual.

Hydrogenation of butter fatty acids has either no effect on their growth-promoting value or else it reduces it. In the absence of other fats hydrogenation does not improve growth, even in the presence of relatively large amounts of lactose from the skim milk powder in the Sherman B diet. It seems unlikely therefore that butter owes its growth-promoting effect to a saturated fatty acid.

Desynon was chosen as a source of vitamins A and D so that each rat could be given an identical, approximately known vitamin supplement without using a carrier oil which might influence the absorption of fat-soluble vitamins. The fatty acids used were free of vitamin A and carotene. The stored vitamin of all livers was therefore derived from an equal amount of preformed dietary vitamin A, and any difference in storage should be expected to be due to differences in vitamin A metabolism. As there was no significant difference between the amount or concentration of vitamin A stored in the livers of animals receiving either butter fatty

acids or their completely hydrogenated counterpart, it is not considered likely that unsaturated fatty acids in general or a specific unsaturated fatty acid present in butter fat have a specific influence on the absorption and storage of vitamin A. This does not preclude the possibility that they may in some way effect the utilization of carotene.

SUMMARY

1. There was no statistically significant difference in weight gain or efficiency of food conversion during a 5-week period between 2 groups of 10 young rats (5 males and 5 females) each, regardless of whether the fat component of a Sherman B diet was made up of the fatty acids of butter or the same fatty acids, stripped of their low molecular weight fraction by steam-distillation.

2. In a similar experiment a significant difference in weight gain and some difference in the efficiency of food conversion was observed when the effect of butter fatty acids as part of the Sherman B diet was compared with that of the partially hydrogenated fatty acids. Weight gain and efficiency of food utilization were decreased only in the male rats of the group fed the hydrogenated fatty acids, and only when the intake of vitamins A and D was relatively low.

3. The amount and concentration of vitamin A stored in the livers of rats fed preformed vitamin A was not significantly affected by complete hydrogenation of the butter fatty acids in the diet.

ACKNOWLEDGMENTS

The author gratefully acknowledges help and advice given him during the course of this work by Dr. W. R. Bloor and Dr. A. B. McCoord.

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MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1948 Award of $1,000 established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the Spring of 1948.

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To be considered by the Committee of Judges, nominations for this award for work published in 1947 must be in the hands of the Nominating Committee by January 10, 1948. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

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